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# *Bacteriological Reviews*

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# THE INHIBITION OF REPRODUCTION OF PARASITES BY IMMUNE FACTORS<sup>1</sup>

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First of all, I should like to express my great appreciation for the invitation to give this lecture. I should have considered it a great honor to give any one of this newly organized series, but I feel particularly honored in being selected to give the lecture named after Ludvig Hektoen. Beside my respect for Dr. Hektoen as a scientist, which I share with all of you, I count among the great benefits of moving to Chicago the fact that it has given me some twenty years of his friendship and gracious advice.

Today, I should like to present some of the work on the inhibition of reproduction of the animal parasites in the body by immune factors. I hope to demonstrate some of the advantages of the protozoa and worms for this type of study. The subject has been of interest to me and my co-workers for about 25 years, but it is still a very incomplete picture.

Although immune factors are frequently assumed to inhibit the rate of reproduction of invading organisms *in vivo*, there are only a few instances in which such an inhibition has been unequivocally demonstrated. The difficulty of demonstrating it arises chiefly from two facts. In the first place, most antibody effects result in the fairly rapid death of organisms *in vivo* or their quick removal from the body, or both, whereas an antibody effect on the rate of reproduction takes time and necessitates observations over periods sufficiently extended to demonstrate an inhibition of reproduction. In the second place, where there are no easily recognizable morphological changes due to reproduction, conclusions must be based on changes in the number of organisms. In such cases, a static population is generally interpreted as indicating an inhibition of reproduction whereas such populations may and, especially in malaria, can at times be shown to result from a nicely balanced reproductive rate and death rate of the parasite.

Neither difficulty holds for some of the animal parasites. They can be comparatively easily found in the host, and their reproduction, provided sufficient organisms survive, can be measured directly. Thus, a measure of the reproductive activity of nematode worms can be based on fecundity or the egg-producing capacity of the females. In certain malarial parasites, more or less synchronous reproduction permits a direct measure of the rate of reproduction which is independent of the number of organisms destroyed, and the localization of the parasites to the blood stream allows approximate determinations of the changes of total population by ordinary hematological methods. Various trypanosomes also possess the advantage of being more or less evenly

<sup>1</sup> An amplification of the Ludvig Hektoen Lecture delivered before the Society of American Bacteriologists, Philadelphia, Pennsylvania, May 15, 1947

distributed in the blood, and, although they do not reproduce synchronously, their reproduction can be gauged by the presence of dividing and variable forms due to division and growth.

All inhibition of parasitic reproduction by immune factors must ultimately rest on the impairment of metabolic activities of the parasite. So far, at least two and possibly three types of reproductive inhibition have been found. In the first type, which occurs in the nematodes, immune factors apparently reduce the entire metabolic level of the parasite, including those processes which are involved in reproduction. In the second type, which occurs in some of the malarial organisms, immune factors act similarly but are probably fortified by physiological derangements of the host resulting from the general toxicity of the antigen-antibody reaction. In the third type, which takes place in certain nonpathogenic trypanosomes, immune factors so specifically inhibit reproduction that most of the general metabolic activities of the parasite seem to be carried on without obvious impairment.

1. *The Nematodes: Nippostrongylus muris*. The first type of inhibition of parasitic reproduction by immune factors is well illustrated by the small hook-worm-like nematode, *N. muris*, of the rat. The mechanism of acquired immunity is probably known better in this species than in any other form (see reviews in Chandler (20) and Taliaferro (53, 55)).

The life cycle of *N. muris* is more or less similar to that of many intestinal nematodes and involves both free-living and parasitic stages. The free-living part of the life cycle begins when eggs are passed in the feces of the host. The eggs develop into infective larvae in about a week. The parasitic phase of the life cycle begins with the penetration of the skin by infective larvae and lasts about 2 weeks in non-immune rats. The larvae rarely remain in the skin longer than a day and generally reach the intestine in from 2 to 3 days after migrating through the skin, blood, lungs, trachea, buccal cavity and esophagus. After reaching the upper part of the intestine, the worms develop into adults which measure from 4 to 6 mm in length, and the females generally begin to lay eggs 6 to 7 days after infection. Most of the adult worms are expelled from the intestine approximately 2 weeks after penetration of the skin due to the acquisition of immunity.

Africa (1) and Schwartz, Alicata and Lucker (43) first reported that rats, after recovery from infection with *N. muris*, are relatively immune to a second infection. The cellular and humoral mechanisms of this immunity have been studied from many angles. Suffice it to say here that in non-immune rats, the organisms feed and move freely in the skin, lungs and intestine without deleterious effects to themselves. In the intestine, they feed periodically by piercing the mucosa. In sufficiently immune rats, on the contrary, larvae which penetrate the skin are progressively prevented from feeding and are immobilized and delayed in their migration. Those which are not killed finally reach the intestine but are stunted and are rapidly eliminated. The adult females produce and lay fewer eggs per day and for a shorter period. Schwartz, Alicata and Lucker (43) and Chandler (17, 18) have particularly stressed the retardation of growth and development and the inhibition of egg-laying of the females with-

out, in most instances, any marked lethal effect. Reproduction of the worms, therefore, is markedly inhibited in immune rats.

All of the evidence indicates that the inhibition of growth and egg-laying is the result of an antibody acting as a precipitin. Thus, Sarles and Taliaferro (42) and Chandler (19) were able to transfer the immunity passively. Moreover, Taliaferro and Sarles (59) have demonstrated in their histological studies that the precipitin does not react with the general body surface of living worms but forms precipitates *in vivo* with the excretions and secretions pouring out of the orifices of the parasite, i.e., the mouth, excretory pore and anus. At times, the mouth of the parasite is capped and the whole gut is filled with immune precipitate. Later, after the worm begins to degenerate, antigens apparently diffuse through the cuticle and precipitate forms around the entire worm. All of the phenomena seen in the actively immune animal can be duplicated in the passively immune rat although the various reactions are less intense (60). The immobilization of the worms and formation of precipitate can also be duplicated *in vitro* (Sarles, 41).

Antibodies acting as precipitins are probably functional in immunity against several metazoan parasites, including a number of nematodes, ticks and fly larvae (Blacklock, Gordon and Fine (10), Trager (66), and review in Taliaferro (55)). In some cases, they may largely inhibit growth and reproduction as they do in *Nippostrongylus*. In other cases, they probably kill larger portions of the population.

Various hypotheses have been formulated as to how the precipitins act. Blacklock, Gordon and Fine (10), in their work on the larvae of the myiasis-producing fly, *Cordylobia anthropophaga*, first described such precipitates in the gut and around the larvae in the skin of immune guinea pigs. They assumed that the precipitate prevents the assimilation of food and leads to the death of the larvae by mechanically blocking the gut. Chandler (18) considered this explanation possible but alternatively suggested that the antibodies are anti-enzymes which inhibit the activity of worm enzymes instrumental in digesting and assimilating host proteins. His idea of anti-enzyme antibodies is similar to that of Ascoli's (3) antiblastic immunity in anthrax (*vide infra*). Chandler specifically homologized the anti-enzymes he postulated with ablastin (see later discussion of ablastin, and Taliaferro (53)).

No matter how the antibodies act, it is amply evident that reproduction of such nematodes as *Nippostrongylus* is markedly inhibited. The resulting inhibition of reproduction is not specific, but seems to be simply an expression of a general lowering of the metabolic processes. It is not unlikely that the precipitins act on certain enzymes or enzyme substrates. In either case, they may not specifically inhibit enzyme activity but may simply reduce the effective surface relationship between enzyme and substrate. (See review in Taliaferro (53) for a discussion of the mode of action of precipitins in immunity to nematodes; Dukes (25) and Smith and Lindsley (46) for examples of antibodies affecting bacterial enzymes; and general reviews in Treffers (67) and Sevag (44)).

Immune serums against *Trichinella spiralis* also act as precipitins and have been shown by Oliver-González (35) to be particularly active *in vitro*. Moulder

(unpublished work) has found that anti-trichina immune serum, before it produces any morphological effect on the worms, significantly reduces the oxygen uptake of the larvae utilizing glucose in a Warburg respirometer. This result might have been predicted but is important in view of the more specific inhibition of reproduction to be described later.

**2. The Malarial Parasites.** The second type of inhibition of parasitic reproduction is found in malaria. It is temporary in nature, and its antibody basis has not been established. The plasmodia allow a particularly accurate measure of the rate of parasitic reproduction.

In 1922, W. H. and L. G. Taliaferro (61) began their studies on differentiating reproduction-inhibiting from parasiticidal factors in immunity. For this work, plasmodia and trypanosomes were selected because, since they are restricted to and more or less evenly distributed throughout the blood stream, the course of their infection can be easily followed by means of frequent blood samples without sacrificing the host. Further progress in this work rested upon developing measures of the rate of parasitic reproduction which are independent of the number of parasitic progeny which die. L. G. Taliaferro (49) pointed out that, for the malarial parasite which has a synchronous asexual reproduction, the length of the asexual cycle and the average number of daughter forms, merozoites, produced by each mature form (suitably corrected, when necessary, for the proportion which grow into sexual stages) give a direct measure of the time necessary for a parasite to grow and produce a given number of progeny and, thus, give a measure of the rate of reproduction. She concluded that the mean number of merozoites produced by segmenting parasites was equal throughout the infection, but she did not make determinations at closely spaced intervals. Boyd and Allen (13) and particularly Boyd (12) showed that the number of merozoites produced per segmenter varies throughout the infection (cf., fig. 2).

The use of the synchronous asexual cycle in conjunction with the number of merozoites per segmenter as a method of measuring the rate of reproduction is well illustrated in infections of *Plasmodium brasilianum* in Central American monkeys as shown by Mrs. Taliaferro and me (62, 63). In this species, it takes three days for one parasite to grow up and produce about ten progeny as may be seen in the top right hand corner of figure 1. The length of the reproductive cycle is also shown in figure 1 by the 3-day peaks in the percent of forms (schizonts) with 5 or more nuclei. This rate of reproduction is generally maintained at a constant rate throughout the infection except when there is a high parasitemia and a sharp parasite decline or crisis terminating the acute rise of the parasitemia.

The parasitemia of an infection with an intense immune reaction at the crisis, as shown by the heavy solid line in figure 1, consisted of an acute rise of the infection (24th to 36th day) with a progressive increase at each segmentation, a parasite decline or crisis (36th to 41st day) with an abrupt decrease, and a developed infection (42nd day on) during which the parasitemia remained at a fairly constant low level.

As may be seen in figure 1, during the acute rise of this infection, the rate of

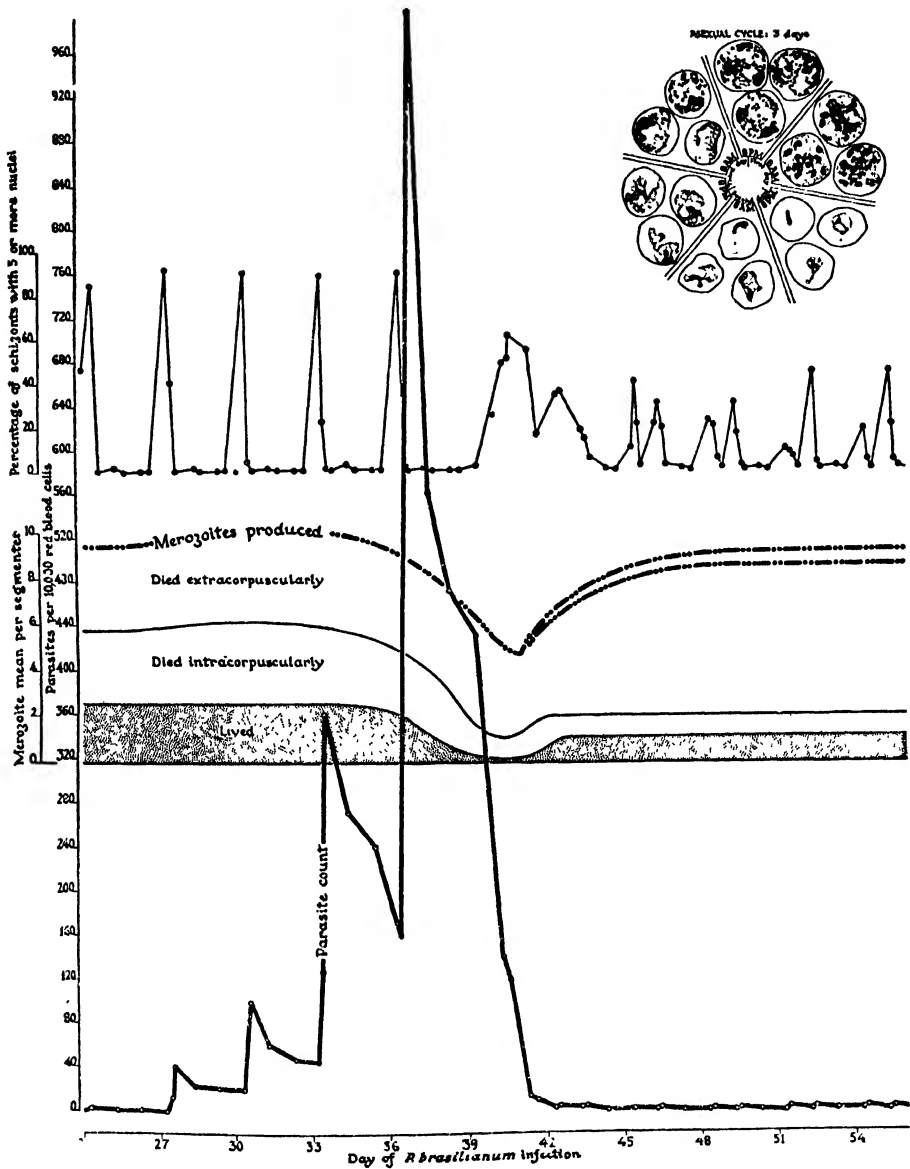


FIG. 1. THE RATE OF REPRODUCTION AND DEATH OF PARASITES DURING THE COURSE OF THE PARASITEMIA OF *Plasmodium brasilianum* IN A CEBUS MONKEY

The asexual cycle is shown in the upper right corner. The rate of reproduction is indicated by the occurrence of schizonts with 5 or more nuclei in conjunction with the merozoite mean per segmenter (merozoites produced). The number of merozoites which live and develop are computed from the net rate of rise in the parasitemia for each segmentation. The merozoites which die can be divided between those which die intra- and extra-corporeally.

Note that a drop in the parasitemia (crisis, 39th to 42nd day) occurs when less than one merozoite per segmenter lives and completes its development and that a static population occurs (after the 42nd day) when approximately one merozoite per segmenter lives and completes its development. (Schematic curves from infection shown in (63), graph 3.)



reproduction was constant at about 10 merozoites per segmenter per 3 days, but, as the exponential rate of net increase in number of parasites was only about 2.5 times per 3 days, it follows that approximately 7.5 merozoites produced per segmenter died. Further analysis of the parasitemia curve indicates that this death took place both extra- and intracorpuseularly. Thus, just after segmentation during the acute rise, the parasites increased by a factor of approximately 6. Hence, on the average, 4 out of 10 extracorpuseular parasites died between segmentation and their penetration of new blood cells. During the 3-day intracorpuseular development, the parasitemia gradually decreased so that the net increase was only 2.5 times the value before segmentation with a consequent average death rate of approximately 3.5 intracorpuseular parasites. The parasites which die are phagocytosed by the macrophages of the spleen, liver and bone marrow (Taliaferro and Cannon, 56). Since both the rate of reproduction and death rate are constant during the acute rise, the parasitemia increases at an exponential rate.

As soon as less than one parasite survives per segmenter, the parasitemia decreases and the crisis ensues. At this time, reproduction may be irregular and markedly inhibited (fig. 1).

During the developed infection, synchronism and the original rate of reproduction are resumed. Frequently, however, the derangement of reproduction at the crisis leads to the occurrence of two broods of parasites whereas only one brood existed before, as is shown in figure 1 by one peak of multinucleated forms every 3 days before the crisis and 2 peaks every 3 days after the crisis. During the developed infection, only one merozoite survived to complete its development, and a static population resulted. Here is an example of a static population in which uninhibited reproduction of the parasite is taking place as shown by the growth and regular division of 2 broods of parasites (fig. 1, top).

From the foregoing discussion, it is evident that parasitic reproduction in *P. brasilianum* may be markedly inhibited at the intense reaction of the crisis. This inhibition, however, is temporary in that reproduction is resumed at its usual rate immediately after the crisis even though the animal is highly immune to superinfection.

Boyd and Allen (13), who first described this type of inhibition of reproduction in *P. cathemerium* in the canary, have suggested that it has an athreptic basis. I believe, however, that this temporary inhibition of reproduction is connected with acquired immunity as indicated by the following facts: (a) It occurs at a time of intense agglutination and filtration of the parasites in the spleen and of intense opsonification and phagocytosis by the macrophages of the spleen, liver and bone marrow. (b) It is associated with crisis forms in the circulating blood, i.e., morphologically degenerate forms prevalent at the crisis.

The question immediately arises, however, why is the original rate of reproduction resumed during the developed infection when the animal is still highly immune to superinfection? This may be due in part to a decrease in antibody titer. We are obtaining more and more evidence that immunity is strongest at the parasite decline or crisis and gradually decreases thereafter. It is probably also due in part to toxic effects of the intense antigen-antibody reaction at the

crisis. These effects are manifested by such changes as an irregular fatty change in the liver (Taliaferro and Cannon, 56) and sickness of the monkey such that it becomes sluggish, doesn't eat, and its temperature drops. Indeed, some animals die. This idea is supported by the work of Stauber (47) which indicates that such factors as altered body temperature and host activity markedly derange the asexual reproductive cycle.

In blood-induced infections with *P. gallinaceum* in the chicken, the number of merozoites produced per segmenter and, therefore, the basic rate of reproduction starts at a high level and steadily decreases throughout the acute rise until it reaches a low point at the end of the crisis (fig. 2). Working with this parasite, Moulder and I (unpublished work) have been unable to demonstrate that supposedly immune serum inhibits the oxygen uptake of parasites, either within the red cell or freed from it except in a few cases when the parasites are agglutinated. These negative results may mean nothing because antibodies have only been sporadically demonstrated in this infection. They may, however, be significant and indicate that the toxicity associated with the antigen-antibody reaction is in reality the major factor even though it is not readily demonstrated. Furthermore, the agglutination seen in occasional serums may be similar to the *in vivo* agglutination during the parasite decline or crisis and may represent the direct action of antibodies on the parasites. If so, the inhibition of oxygen uptake would result from a mechanical reduction of the effective surface contact between the parasite and the medium. In so far as the antibody acts directly on the parasite, malaria would resemble *Nippostrongylus* infections in which the precipitin acts on the worm. Thus, agglutination might cause a reduction in surface contact between the organisms and the medium, which in turn might easily cause a generalized suppression of metabolic activity including those processes necessary for reproduction. It may be noted, however, that Sevag and Miller (45) found no diminution of oxygen consumption in *Eberthella typhosa* and pneumococci while agglutinated by immune serum.

The interrelations of parasite reproduction and survival present a fascinating picture and are shown diagrammatically for six infections in figure 2. In this figure, stages of the various infections are shown as if they were of the same length, and detailed parasitemia curves are omitted. In a typical infection by *P. brasilianum*, as contrasted to that shown in figure 1, which had an exceptionally marked immune reaction, the rate of reproduction is maintained at a constant level (approximately 10 are produced every third day) except for a temporary decrease at the parasite decline during some infections, whereas the survival of parasites is constant (around 3) during the acute rise, decreases at the crisis, and then is again constant during the developed infection (although at a lower level—around 1—than during the acute rise) except for sporadic temporary increases during relapses (63).

Infection with *P. lophurae* in the chicken is essentially similar to *P. brasilianum* except that the initial rate is higher (11 merozoites are produced in half the time) and the survival rate (around 4) is higher during the acute rise (W. H. and L. G. Taliaferro, 65, and unpublished work).

In infection with *P. cynomolgi* in the rhesus monkey, the rate of reproduction

progressively decreases during the acute rise until the parasite decline is reached, as shown by a decrease from 15 to 9 in the merozoites produced, then rises and is maintained at an essentially constant level (around 15) during the developed infection. The survival of the parasites is practically 100% at the beginning of the infection but sharply decreases during the acute rise to an extremely low

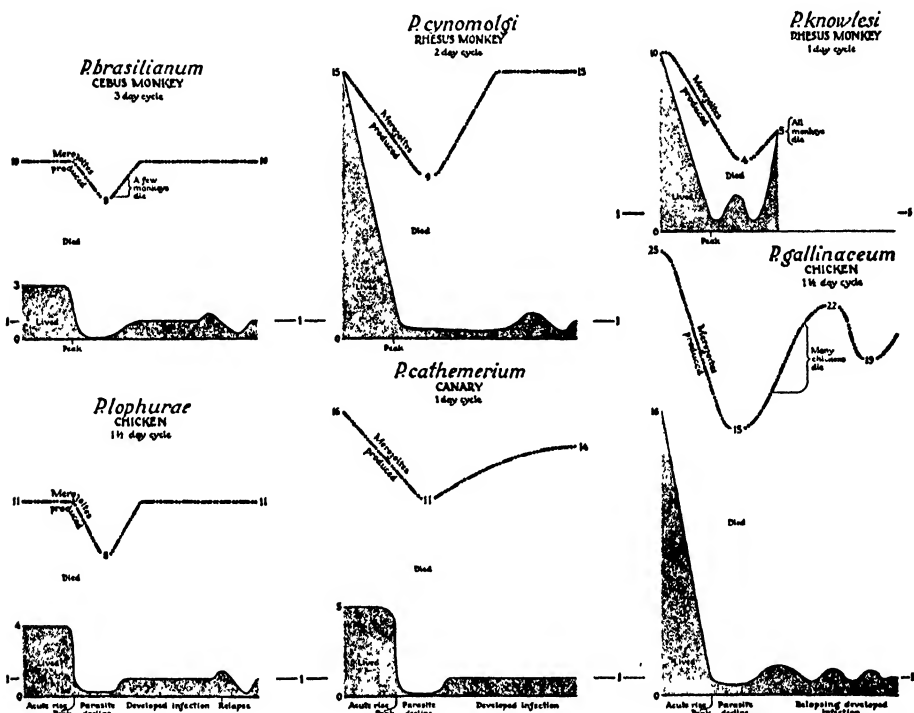


FIG. 2. SCHEMATIC DIAGRAMS OF THE RATE OF REPRODUCTION AND THE SURVIVAL AND DEATH RATES OF SEVERAL SPECIES OF PLASMODIA

The rate of reproduction is shown by the merozoites produced (since the asexual cycle is usually constant) and the death rate is the difference between the forms that are produced and those that live.

Note that, for purposes of comparison, the stages of infection, i.e., acute rise, parasite decline and developed infection, are arbitrarily indicated by the identical intervals in each infection, although they vary over a wide range. (Graphs are based on data of *P. brasilianum* from (63); *P. cynomolgi* from (64); *P. gallinaceum* from (65); *P. cathemerium* from (49), (12), and unpublished work; *P. lophurae* and *P. knowlesi* from unpublished work).

point (less than 1 merozoite) during the parasite decline, and thereafter only temporarily and slightly increases (W. H. and L. G. Taliaferro, 64).

In infection with *P. cathemerium* in the canary, changes in the rate of reproduction are somewhat similar to those encountered in infection with *P. cynomolgi*, whereas the survival rate is somewhat similar to that encountered in infection with *P. brasilianum* and *P. lophurae* except that relapses are uncommon (L. G. Taliaferro (49), Boyd and Allen (13), Boyd (12)). In this infection, we have the anomaly of a generally decreasing rate of reproduction with an essentially

constant survival rate during the acute rise of the infection (Boyd and Allen (13), Boyd (12), and unpublished work by W. H. and L. G. Taliaferro).

In the first part of the fatal infection with *P. knowlesi*, changes in the rate of reproduction and survival of parasites follow the general pattern seen in infections with *P. cynomolgi*, but there is no pronounced crisis and survival of the merozoites increases to 100% just before death (W. H. and L. G. Taliaferro, unpublished work).

Finally, the infection of *P. gallinaceum* in the chicken is somewhat similar to the infection of *P. knowlesi* except that the rate of reproduction is higher throughout (15 to 25), the initial survival of the parasites at the beginning of the infection is less than 100%, and the waves of increased survival are comparatively small (W. H. and L. G. Taliaferro (65) and unpublished work). Although observations on *P. gallinaceum* were made on blood-induced infections in which exoerythrocytic stages are relatively infrequent during the acute rise, the foregoing analysis may be invalidated to the extent that exoerythrocytic stages do occur, particularly after the crisis.

3. *Trypanosomes of Rodents: Trypanosoma lewisi*. The third, and, to me, the most interesting type of inhibition of parasitic reproduction has so far been demonstrated unequivocally only in infections with trypanosomes belonging to the *T. lewisi* group. It is brought about by an antibody which is so exquisitely specific in action that it inhibits reproduction with no apparent effect on the general vitality, motility, or infectivity of the organisms.

*T. lewisi* is a relatively non-pathogenic blood parasite of rats all over the world and is transmitted from rat to rat by various species of fleas. It is representative of a large group of trypanosomes which occur in various rodents. All have approximately the same morphology and life cycle, but each is highly specific for its vertebrate host.

The development of the parasitemia in rats following experimental infections induced with infected blood has been described by Steffan (48) and subsequent observers (61, 50, 22, and review in 54). Immediately after the injection of blood there is a prepatent period, which may be of several days' duration or which may be lacking if sufficient organisms are injected. Once the organisms are seen in the blood, their numbers increase and may reach a peak of 300,000 per cmm or more 4 to 7 days later. Once the peak is reached, the parasites may be removed from the blood rapidly in a crisis-like decline or gradually by a series of small decreases. Sooner or later, they reach a somewhat constant number (developed infection) but eventually disappear. Thereafter, the rat is more or less immune to a second infection.

As early as 1899, Rabinowitch and Kempner (37) reported that the blood of rats during the early part of their infection contains many dividing forms whereas later in the infection, it contains only non-reproducing parasites. This conclusion was fully verified by von Wasielewski and Senn (68), Laveran and Mesnil (28), MacNeal (29), Brown (14) and especially by me and my coworkers who applied statistical methods to the problem (Coventry, 22; W. H. and L. G. Taliaferro, 61; Taliaferro, 50). We have found that all mitotic division and

growth is generally completely inhibited by the tenth day of the infection and sometimes earlier.

For these studies and especially for demonstrating an antibody basis, methods of measuring the rate of reproduction are necessary which are independent of how many parasites die. Two basic methods are now in use. The first of these relies upon the occurrence of dividing trypanosomes. The early investigators (37, 68, 28, 29, 14) were content to note the occurrence of dividing forms in blood smears, and this method is still accurate for determining whether or not reproduction is occurring (see 57). Later workers determined the percentage of dividing forms, variously defined, during the course of infection (see Robertson (40) and Krijgsman (27) for pathogenic forms; and Taliaferro and Pavlinova (58) for *T. lewisi*). The second method involving the coefficient of variation was devised by Mrs. Taliaferro and me (61). It depends upon the fact that members of a growing population vary in total length because of young and growing forms, whereas those of an adult population do not. Both methods have certain advantages (58).

The inhibition of reproduction of trypanosomes is brought about by an antibody which I (50) demonstrated in 1924 and later called ablastin (51). Disappearance of the trypanosomes can frequently be associated with a typical lysin which can be complemented with fresh guinea pig serum (51, 52). It may, however, act chiefly as an opsonin (4), possibly, in part, because the rat is deficient in lytic complement.

Ablastin as well as the lysin is specific in action, is precipitated in the globulin fraction of immune serum, and arises after immunization with killed organisms (see 51). As first shown by Regendanz and Kikuth (38), the formation of ablastin is lessened by splenectomy. This antibody has been studied by a number of investigators from several aspects (see review in 54).

Ablastin differs from the lysin in the firmness of its union with trypanosomes (51). If lytic serum is absorbed with either dividing or adult trypanosomes, not only is the lysin removed, but the trypanosomes are sensitized so that they quickly disappear from the peripheral blood when injected into normal rats. In marked contrast, when ablatic serum is treated repeatedly with dividing and adult trypanosomes, ablastin is not removed. This lack of absorption cannot be due to an insufficient number of trypanosomes because the parasites used for absorption are not sensitized, i.e., they are not prevented from dividing when put into normal rats. Actually, however, if sensitization occurred, a blood infection could never be transferred to a normal rat except during the first few days when the organisms are not inhibited by ablastin. This characteristic may differentiate all true ablatic types of antibodies (see discussion of nonabsorbable antibodies in helminth infections, 15, 16, 36, 53).

Recently, Augustine (4) concluded that recovery from reinfection with large numbers of parasites involves only the trypanocidal antibody and that ablastin plays no rôle. He states that the parasites may remain for varying periods without increasing in number even though they are dividing because of a differential susceptibility of the dividing forms to the trypanocidal antibody.

I accept entirely the results of Augustine and consider them to be important extensions of previous findings by Coventry and me. In passively transferring the trypanocidal antibody, I have frequently seen static populations containing dividing trypanosomes. They probably also occur in superinfections because, as Coventry (22) first demonstrated, ablastin decreases in titer as the infection proceeds. The large numbers of trypanosomes which Augustine used would, therefore, be expected to overwhelm the weaker ablastin before it did the stronger lysin. However, Augustine's work can be interpreted as showing that there is no such antibody as ablastin (see Kidd (26), Dubos (24), Becker and Gallagher (8)). In other words, a static population of adult trypanosomes would be brought about by a differential removal of dividing parasites. I cannot accept this view because, in the first place, it is difficult to conceive of an infection in which dividing parasites are removed so completely that they are never seen. In fact, Augustine saw dividing forms in his reinfections although they are not seen in the latter part of initial infections. In the second place, such a view contains an inherent contradiction in that a population, in which adult trypanosomes were

TABLE 1\*  
*Oxidative metabolism of Trypanosoma lewisi*

REPRODUCTIVE STATE OF TRYPANOSOMES	DIVIDING AND GROWING	NON-DIVIDING ADULTS	DAY OF INFECTION ON WHICH CHANGE OCCURRED
Oxygen uptake $\mu\text{M}/10^9$ trypanosomes/2 hr. . . .	45.	62.	5
Glucose utilization $\mu\text{M}/10^9$ trypanosomes/2 hr. . .	42.	25.	4
Oxygen/glucose ratio. . . . .	1.03	2.78	4
Respiratory quotient. . . . .	0.74	0.91	4

\* Modified from Moulder (34).

removed as soon as they started to divide or even after they had divided, would not remain stationary but would decrease.

Moulder (32, 33, 34) has begun a study which promises to furnish considerable evidence on the mechanism of ablastic action. In preliminary studies, he has found that ablastic serum has no effect on the oxygen uptake of dividing *T. lewisi* utilizing glucose in a Warburg respirometer (personal communication). Such a result might be expected because of the comparatively short time the serum is acting on the parasite in such experiments. On the contrary, he (32, 34) has obtained positive differences when trypanosomes are observed at different stages in the infection, i.e., when they are rapidly dividing (before the 4th or 5th day of the infection), and after their reproduction has been inhibited by ablastin *in vivo* (after the 5th day of the infection). As shown by the data for the dividing as compared to non-dividing forms in table 1, trypanosomes whose reproduction was inhibited had a higher oxygen uptake, oxygen/glucose ratio and respiratory quotient, and a lower glucose utilization (34).

He points out that the effects of ablastic action *in vivo* are similar to two types of previous findings. Thus, in work on the effect of such inhibitors as urethane, azide and dinitrophenols upon respiration and cell division in yeast cells and sea

urchin eggs, Fisher and Stern, Fisher and Henry, and Krah1 and Clowes (see review by McElroy, 30) have found that low concentrations of these inhibitors inhibit cell division with only a slight depression or even a stimulation of oxygen consumption. In addition, Barker, Giesberger and Clifton (see review in 21, 30) have shown that cells not only oxidize substrates for energy but also oxidatively assimilate part of the substrates. In the latter process, more substrate disappears than can be accounted for by its complete oxidation to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , and, therefore, both the respiratory quotient and the oxygen/substrate ratio is low. Low concentrations of such inhibitors as azide and dinitrophenols may inhibit oxidative assimilations without depressing oxygen consumption just as is the case when they inhibit cell division. Under such conditions, the R. Q. rises and oxygen consumption can be largely accounted for by the complete oxidation of the substrate which disappears. Moulder suggests that ablustin may inhibit cell division and growth by inhibiting the oxidative assimilation of glucose in reproducing trypanosomes in a manner similar to the inhibition by azide or dinitrophenol in concentrations too low to reduce the rate of oxygen consumption. Thus, young reproducing trypanosomes oxidize glucose incompletely because they are oxidatively assimilating glucose whereas adult non-reproducing trypanosomes oxidize glucose more completely because the oxidative assimilation has been inhibited.

Moulder (32, 34) further found that, although the degree of inhibition is never great, sodium malonate more markedly inhibits the oxygen uptake of non-reproducing adults than of dividing trypanosomes. This would suggest that the suppression of oxidative assimilation under the influence of ablustin results specifically from the loss of the power to carry oxidation past the succinic acid step in intermediary metabolism. However, Moulder was unable to relieve the malonate inhibition of glucose oxidation with fumarate or to obtain the oxidation of added succinate in either young or dividing trypanosomes. Similarly, Reiner, Smythe and Pedlow (39) reported that succinic acid is one of the end products of aerobic metabolism in this species.

Becker and his associates (7, 9) have found that the reproductive phase of the infection is prolonged when pantothenic acid is withheld from the host's diet or when sodium salicylate is administered *per os* (8). As a result of these findings, Becker and Gallagher (8) suggested that ablustin is an oxidative enzyme composed of a protein moiety in combination with pantothenic acid. According to them, the apoenzyme is a specific protein which accumulates in the blood during immunization and becomes associated with the coenzyme, pantothenic acid, normally present. They thus explain the effects of pantothenic acid deficiency by the absence of the coenzyme, and the action of salicylate by its combining with the protein in place of the coenzyme.

As far as infections produced by relatives of the lewisi-group of trypanosomes have been studied, they are characterized by the eventual inhibition of reproduction and hence, inferentially by the formation of ablustin. The infection of the mouse with *T. duttoni* is interesting because, besides an acquired ablustin, the mouse possesses an innate ability partially to inhibit reproduction of *T. duttoni*

(58). This innate factor cannot be passively transferred and has not so far been demonstrated in a normal rat against *T. lewisi* (52, 54).

4. *Related Studies.* There are several reports in the literature of immune serums inhibiting the reproduction of bacteria within the body. At the present time, however, such an antibody action has not been definitely established *in vivo* and probably cannot be as firmly established as it has been for some bacteria in the presence of certain dyes or drugs in appropriate concentrations. The difficulty involves the differentiation of a population of viable, non-reproducing cells from one in which active cell division is occurring but is just balanced by the rate of death. In fact, advances in establishing reproduction-inhibiting immune factors may only be possible after we know the metabolic pathways, and the specific action of immune factors on these pathways. I would, however, like to call a few of the more important studies to your attention.

Historically, the most important is the concept of antiblastic immunity which was developed by Ascoli in 1906 and 1908 (2, 3) while working on anthrax in the guinea pig. Parenthetically, it should be noted that I was unaware of this work when I proposed the term ablazin. Ascoli concluded that the protective action of serum is due to the inhibition of certain assimilative processes of the bacteria which prevents germination and is associated with a delay in capsule formation. He further found that this antiblastic principle is nonabsorbable with specific bacteria and does not kill them. Later, when ablazin in *T. lewisi* infections was found to possess these same characteristics, Ascoli believed the antibody he had described to be identical with ablazin (3a). At present, the data seem inadequate to decide whether Ascoli's principle works in the specific way I have described for ablazin. On the other hand, there is not sufficient evidence to disprove it.

Dochez and Avery (23) believed that antiblastic factors occur in antipneumococcus serum which are anti-enzymatic in nature and cannot be explained by agglutination. Later, however, Blake and Barber questioned this. Using single-cell methods, Barber (5, 6) could not obtain any evidence of the inhibition of growth or lengthening of generation time of pneumococci under the influence of immune serum either *in vitro* or *in vivo*. Blake (11) found that immune serum inhibits various metabolic activities of the pneumococcus, but that this inhibition varies with the agglutinin content and disappears after removal of the agglutinins by absorption. The agglutinins act apparently in much the same manner as suggested for precipitins against nematodes in that they decrease the surface contact of bacteria and medium (cf. the negative results on respiration reported by Sevag and Miller, 45).

Campbell (15, 16) has used the property of nonabsorbability to differentiate two types of protective antibodies against the larval form of the tape worm, *Taenia taeniaeformis*, and to homologize one with ablazin. Here again more detailed studies, especially as to the effects on metabolism, will be necessary to prove the similarity or dissimilarity of the two.

Finally, of great interest is the description by Kidd (26) in 1946 of an antibody, produced by rabbits implanted with the Brown-Pearce tumor or injected



with cell-free extracts of it, which is capable of suppressing the growth of the tumor cells *in vivo*. He could observe no microscopic effects of the immune serum on the cells *in vitro*, such as lysis, agglutination or abnormal staining reactions. To analyze such data with respect to reproduction, one prime condition can be satisfied, i.e., mitotic division can be adequately differentiated from cellular destruction just as in the protozoa. The same would be true of tissue cells if the recent suggestion of Medawar (31) is verified. Medawar believes that the destruction of homologous skin grafts in rabbits is due to the action of antibodies which prevent nuclear division in the cells of the grafted tissue.

*Conclusions.* In conclusion, I should like to stress the fact that most antibodies either kill parasites quickly or effectively aid in rapidly removing them from the body. There are relatively few proven conditions in which antibodies inhibit the basic rate of reproduction of the parasite *in vivo*. Many alleged cases are based on the occurrence of a static parasite population but, in such a population, the members may actually be multiplying and may be being killed by lysis or phagocytosis at the same rate that they are being produced.

There are, however, a few infections in which reproduction of the parasites can be shown to be inhibited. In some of these, the same antibody which kills and aids in eliminating the parasites from the body, also partially inhibits reproduction of the parasites. Thus, a precipitin in worm infections does not specifically inhibit reproduction but probably depresses all metabolic activities—including those on which reproduction rests. In malaria, a somewhat similar nonspecific type of inhibition of reproduction occurs and is probably fortified by the toxicity of the intense antigen-antibody reaction at the crisis which primarily deranges the host's activity and secondarily the parasite's reproductive cycle. The antibody bases for these effects have not been established.

Finally, in infections with *T. lewisi*, ablastin specifically inhibits reproduction of the parasites with no apparent effect on the general activity or infectivity of the parasites. This antibody seems to form no lasting union with the organism. As inhibition of reproduction occurs *in vivo*, the oxidative processes are modified in a manner which suggests that the oxidative assimilation of the substrate is reduced and that in nondividing trypanosomes most of the substrate is oxidized to supply energy for maintenance. Biologically, this antibody is of great interest and may involve mechanisms related to the suppression of cell division in normal development or to the increased cell division in tumors. Unfortunately, the proved occurrence of ablastin in immunity is limited to one group of non-pathogenic trypanosomes, although it may play a rôle in certain bacterial and tapeworm infections and in transplantable tumors.

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# THE ENDOSPORE OF BACTERIA

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An *endospore* is an intracellular spore formed chiefly by certain bacteria and capable of reaching a high degree of resistance to deleterious agents. This definition implies three points, usually not understood, about the endospore: the first is that the biological significance of the endospore is not yet known and, consequently, that the term should be considered provisional; the second is that endospore formation has been observed outside of the bacteria, *e.g.*, in the genus *Oscillospira* of the blue-green algae (38, 40); the third is that the resistance of the endospores in a given species does not have a fixed value.

In the bacteria, endospore formation is most prevalent among the rod-like forms and is the basis for the anaerobic genus *Clostridium* and the facultative *Bacillus*. However, it is found, although rarely, in other groups. We thus have the endospore-forming *Planosarcina ureae*, *Spirillum amyliferum* (166), and *Spirillum praeclarum* (40).

The importance of endospore formation is two-fold: first, it constitutes the only certain and reproducible cyclostage in bacteria; second, the high resistance which it may attain has determined the bacteriological methods of sterilization. The practical importance of these methods in medicine and in the food industries can not be overestimated.

The endospore appears as a highly refractive body inside of a mother cell usually called *sporangium*. Ehrenberg (54) observed in various protozoa, among which he placed the bacteria, refractile bodies that he called eggs. It is not certain whether any of the forms in which such bodies were observed were genuine bacteria. Perty (133) observed such bodies in undoubtedly true bacteria, expressed the opinion that they might be spores, and used their presence as the basis for a genus he called *Sporonema*. Perty did nothing to prove that the bodies he observed were spores, although examination of his drawings leaves no doubt about the correctness of his supposition. Pasteur (132) was the first to correlate the presence of refractile bodies in bacteria and resistance to deleterious agents. It remained for Cohn (23) and Koch (104) to prove, the first

with *Bacillus subtilis* and the second with *Bacillus anthracis*, that the refractile bodies observed in these organisms may germinate when placed in a new medium and develop from spore to spore.

It is not the purpose of this review to discuss the extensive literature on the resistance of the endospore to deleterious agents. This would require a separate review. We shall merely point out several properties of the endospore which seem to account for its resistance. These are: *a*, the low permeability of the coats which is undoubtedly the chief factor in resistance to chemicals; a fair degree of correlation has also been found between toughness of the coat and resistance of various strains to heat; *b*, the presence of a relatively considerable quantity of ribonucleic acid throughout the cytoplasm of the spore, which undoubtedly protects the protoplasm against ultraviolet rays, electrons, etc.; *c*, the low free water content which should be an important factor in resistance to heat.

Differences in resistance between different spores of the same culture and in the average resistance of the spores in different strains or species may be attributed to differences in one or more of these characteristics. This is probably why resistance is affected by the environmental conditions under which the spore is formed. Also, in a given culture many spores show evidence of incipient germination and, consequently, a corresponding drop in resistance.

Differences in resistance to heat between spores of different strains or species may be somewhat correlated with the maximum temperature of growth. This is undoubtedly due to differences in thermostability of important components of the protoplasm. Indeed, a protoplasm which thrives at 65 C must be different from one that is destroyed below this temperature. There is no evidence, however, for the existence of such a difference between the protoplasm of the vegetative cell and that of the endospore in the same strain.

#### CYTOLOGY

##### *Structure of the resting endospore*

The structure of the endospore has been the subject of numerous investigations promoted by its obvious relation to the structure of bacteria in general, and by its bearing on the problem of resistance. Unfortunately, the endospore is not a good object for cytological investigation. Besides its high optical density and low degree of permeability to dyes and other reagents, it shares with the vegetative cell all the other factors which, for nearly three quarters of a century, made that cell a Gordian knot for the cytologist. Consequently, definite knowledge about the structure of the endospore was, until very recently, in a primitive state.

Cohn (23) considered a spore of *Bacillus subtilis* to consist of a thin, gelatinous layer (the spore coat) surrounding a strongly refractive content. As a result of observing spore germination, Koch (104) believed that the endospore of *Bacillus anthracis* consists probably of oily droplets surrounded by a thin protoplasmic layer; this layer is the living substance, but the oily droplets are probably reserve material to be used up during germination. There is no mention of a

spore coat. Migula (125) explained Koch's inability to see the coat by assuming that in the media used by Koch the organism did not shed a coat upon germination.

Brefeld (10) was the first to observe a double membrane around the spore of *B. subtilis*; the outer one or exosporium (not in the sense used by Lewis, 114) is shed off upon germination, and the inner one becomes the wall of the germ cell. Treatment of the spore with a number of reagents (iodine, zinc chloride + iodine, ether, chloroform) revealed nothing about the internal make-up of the spore. However, since the spore became transparent, particularly at the middle, when treated with concentrated sulfuric acid, Brefeld concluded that it consists of combustible organic material.

According to deBary (36), the spore consists of a homogeneous, strongly refractive protoplasm surrounded by a thin, firm, apparently brittle membrane; this membrane is often surrounded by a pale, weakly refracting, and poorly delimited layer of a gelatinous consistency, which is often drawn into tail-like appendages at the spore ends. Klein (82) stated that the spore of *Bacillus leptosporus* possesses a membrane differentiated into two layers, such as one observes in *B. subtilis*, *B. megatherium*, *B. sessilis*, and other organisms. This sharply delimited membrane is surrounded by a fairly broad, mat halo of slime which is not merely an optical phenomenon, since one never sees two contiguous spores touching each other at their sharply delimited membranes. Burchard (15) observed two coats shed off by the germinating spore of *Bacillus petroselinii*; the inner coat was darker and thicker than the outer one. Two such coats were also illustrated by Migula (123).

An intensive study of the endospore by Meyer (120, 121) led him to conclude that it consists of a protoplasm containing a nucleus and cell-sap vacuoles and surrounded by a membrane in two layers; the outer layer is the episporium or exosporium (in the sense used by Brefeld, 10), and the inner layer is the endosporium (not in the sense used by Lewis, 114). Meyer suggested for these membranes the names *exine* and *intine*, respectively. The *exine* and *intine* form the firm spore membrane which is often surrounded by a slime layer. These three membranes were carefully illustrated in a later publication (122). Meyer also stated that the resting spore may still contain another membrane, that of the germ cell.

The resting endospore of *Bacillus bütschlii*, according to Schaudinn (153), consists of a dense, homogeneous protoplasm surrounded by two coats. The inner coat encloses the protoplasm completely; the outer one leaves bare a small area, a sort of a germ pore, at the outer pole where the germ cell emerges during germination. The homogeneous protoplasm consists chiefly of a mass of chromatin resulting from the fusion of numerous granules which had gathered in the forespore. In *Bacillus sporonema* (154), the spore protoplasm also consists chiefly of chromatin resulting from the growth of a granule, but no germ pore is observed. Guilliermond's view (70) on the structure of the endospore in several spore formers is similar to that of Schaudinn (154) on *B. sporonema*.

Preisz (142), studying chiefly *B. anthracis*, makes no mention of an *exine* or



intine but writes of a spore coat formed by shrinkage of the outer layer of the forespore. It can be concluded from his description of spore formation that the protoplasm of the spore may contain a nucleus, at least during certain stages of its development, but no acid-fast or metachromatic granules. In a later paper (143), he describes the spore as consisting of a shiny body and a homogeneous coat which is uncolored by vital staining; at most, the contour of the shiny body and the coat are weakly stained. Occasionally, one finds between the poles of the shiny body and the contour of the coat round or irregular granules which must be the rest of the chromatic substance concerned with the formation of the spore coat. Almost always, the spore nucleus is located at the lateral boundary of the shiny body, not at one of its poles. The shiny body consists of reserve material surrounded by a thin layer of protoplasm. The wall of the germ cell does not pre-exist in the resting spore but is formed during germination. Preisz observed spores with a different structure, but interpreted the differences as evidence of germination.

Schussnig (156) believed to observe, in an unidentified bacterium, a spore consisting of a strongly refractive membrane with a double contour and a protoplasm containing two nuclei. Petersen (135) stated that the endospore of *B. mycoides* is, in all stages, usually uninucleate. However, when the mother cell contains two nuclei, the second nucleus "later moves towards the young spore and perhaps invades it." Badian (3, 4) reported one, dumbbell-like chromosome in the spore of *B. subtilis*, *B. mycoides*, and *B. megatherium*.

Lewis (114) was unable to demonstrate a nucleus in the endospore of *B. mycoides*. He stated: "There is no conclusive evidence that deeper staining capacity of the spore primordium is due to a difference in chromatic material. On the other hand, such evidence as can be obtained by the stains employed points to the opposite conclusions. The material, except for a single deeply stained granule which is not always visible, has every appearance of uniformly stained cytoplasm." He further adds: "It is probable that the true spore wall is made up of two layers, the outer exine and the inner intine, as in larger and better known spores but this is difficult to demonstrate." The exine is surrounded by a stainable layer, thin at the sides and thick at the poles, which represents the shrunken exosporium (for the meaning in which he uses this term, see below). Delaporte (39-41), studying endospores of various bacteria, observed a refractile membrane, a hyaline cytoplasm, and a Feulgen-positive rod or granule.

The structure of the resting spore, hydrolyzed with normal HCl, was recently investigated by Robinow (148) in the following members of the genus *Bacillus*: *B. mycoides*, *B. mesentericus*, *B. cereus*, *B. anthracis*, and *B. megatherium*. It was found similar in all of these organisms, and was described as follows: "In the cytoplasm of hydrolyzed spores, mounted in water, three concentric layers can be distinguished. The central portion, a narrow core, is refractile and stainable, and merges into a thick layer of less refractile and less readily stainable cytoplasm, bounded on its outer surface by a thin crust of stainable, non-refracting material which may be identical with the spore membrane—No chromatinic matter is found in the cytoplasm."

"The nucleus is the most refracting element in hydrolyzed spores; it is attached to the outer surface of the cytoplasm and readily distinguished from it as a brightly refractile body even in unstained water-mounted preparations. The nucleus gives a strong positive Feulgen reaction, in sharp contrast to the vegetative cytoplasm, and is deeply and brilliantly stained with Giemsa solution and crystal violet . . . . In spores hydrolyzed at 57–60°C, the nucleus contracts lengthwise and increases in thickness, and although it remains clearly attached to the surface of the dormant cytoplasm, it now causes a hump in the formerly unbroken contours of the cell."

"Lateral and full views alike suggest that the nucleus is a biconcave disk with the central depression sometimes extending to part of the perimeter. The shape of the nucleus somewhat resembles that of a fresh human red blood cell."

"The fate of the spore membrane during hydrolysis is also not clear and may vary in different species. In *Bacillus anthracis* and *B. megatherium* the spore membrane after hydrolysis can be seen surrounding the nucleus and cytoplasm, but in hydrolyzed resting spores of other species usually it cannot be identified, although it is plainly visible, before and after hydrolysis, as soon as it is discarded by the young, vegetative bacillus at the end of spore germination."

"Some information on the spore membrane was obtained from electron micrographs of hydrolyzed *Mesentericus* spores . . . . Two kinds of spores were seen in hydrolyzed suspensions, both asymmetrical; in some, the asymmetry consisted merely of a slight hump in the cell's smooth and unbroken contours while in others it was due to a large round body with ragged fluffy outlines and a much lower density than that of the main body of the spore from which it protruded. The round body is the spore nucleus, and the pictures suggest that the spore membrane normally clings very tightly to the cytoplasm and that the nucleus, which lies underneath the spore membrane on the surface of the cytoplasm, contracts lengthwise under the influence of the hydrochl. acid—and thereby lifts up, stretches and finally bursts through the spore membrane which, except for the hole made in it, continues firmly to adhere to the cytoplasm."

The structure of the endospore of *Bacillus cereus*, strain C<sub>3</sub>, was investigated by Knaysi (96). It was found that "Forespores and young endospores stain homogeneously by the Feulgen procedure but mature spores show a positive granule or rod." This was taken to mean that "the nuclear material is diffuse both in the vegetative cell and in the young endospore, with a possible differentiation of a nucleus during maturation of the spore." The possibility that the difference was due to shrinkage was also considered. Evaluation of this conclusion in the light of more recent knowledge will be given below. Records of that investigation show that the boundary of the spore and an indefinite zone surrounding it also give a positive Feulgen reaction. There was no indication that the internal, Feulgen-positive body necessarily occupies an extreme peripheral position in the resting spore as claimed by Preisz (142) and Robinow (148).

Physiological analysis (92) showed that the endospore of *B. mycoides*, strain C<sub>2</sub>, contains a relatively considerable quantity of a reserve material suitable as a source of nitrogen but not of energy.

Very recently, the structure of the endospore of *B. mycoides*, strain C<sub>2</sub>, was

investigated by Knaysi, Baker, and Hillier (99), using the high-voltage electron microscope. In a typical spore, one observes an opaque, central mass, the spore protoplasm, surrounded by two coats; of the two, the inner one is the more rigid, and the outer one the more elastic; the latter resembles the exosporic layer of Lewis (114) and the elastic "capsule" observed by Lamanna (106) in *B. mesentericus*. Differentiation of the inner coat into exine and intine was not usually observed. There was no evidence for the existence of a third, innermost coat which becomes the wall of the germ cell; however, such a wall can be seen as soon as the spore coats are split. The authors consider the possibility that this wall was pre-existent, but invisible, in the resting spore; however, they believed more likely its formation during germination, as previously claimed by Preisz (143). A parallel investigation with the electron microscope (EMU), by Knaysi and Baker (98), in which the same organism was allowed to sporulate in a nitrogen-free medium showed that the forespore contains from 2 to 6 nuclei at first grouped at the poles but later occupying other positions. This work confirmed the existence of a readily utilizable source of nitrogen in the spore; by microchemical tests and physiological analysis it was shown that the material is ribonucleic acid diffuse throughout the cytoplasm. Observation, with the light microscope, of spores which used up their ribonucleic acid in the nitrogen-free medium but failed to germinate, confirmed the general bipolar position of the nuclei; central and lateral nuclei were also observed.

*Parasporal bodies.* Debaisieux (34, 35) observed appendages attached to the ends of the endospores or helicoidal structures surrounding the endospores in bacteria found in the intestines of the larvae of the dipterae *Simulium* and *Ptychoptera*, or in the intestines of the frog. Dutky (48) observed in *Bacillus popilliae*, one of the organisms which cause the milky disease of the Japanese beetle *Popillia japonica*, "a refractile body, which is about half the size of the spore and possesses staining reactions similar to those of the spore." This body is located at the broad pole of the sporangium. The origin of these parasporal bodies and their significance are not yet known. It is interesting to note, however, that they have heretofore been observed chiefly in entomogenous bacteria.

*Discussion.* A study of the literature based on the light microscope and reviewed above shows that the number of coats which surround the spore protoplasm varies, according to the investigator, from 1 to 3 or, possibly 4. In the latter case there would be a slime layer, a double coat shed by most species during germination, and the wall of the germ cell. Of the four coats, the slime layer and the wall of the germ cell are the most debatable. What appears as a broad slime layer may well be an optical phenomenon. Indeed, broad halos of light are always observed around bodies suspended in a liquid less refractive than themselves, and this has often led to erroneous conclusions; for instance, the relatively broad halos which surround dense, intracellular inclusions, have often been considered as vacuoles, and the halo observed by Cohn (22) around the bacterial cell was suspected of being the cell wall. Experimental evidence regarding the nature of the halo which surrounds the endospore is conflicting:

Brefeld (10) considered it an optical artifact, since it is not observed around the empty coat, but Klein (82) thought it was a real structure since contiguous endospores never touch one another at the coats with sharp boundaries. In reality, the two authors were not discussing the same thing. Staining of the free spores with Giemsa's solution reveals a thin, faintly reddish layer around the spore which is distinct from the broad, bright halo surrounding the spore. This thin reddish layer may be an integral part of the spore or merely a film of residual material from the cytoplasm of the sporangium. Certainly, there is no broad slime layer which can be demonstrated with the electron microscope around the spore, free or in the sporangium. A thick slime layer which is not sufficiently dense to be observed around the free spore should, certainly, be demonstrable in the sporangium as a transparent ring between the outer spore coat and the cytoplasm of the sporangium. Published illustrations (99) do not show such a ring.

Pre-existence, in the resting spore, of an innermost coat which becomes the wall of the germ cell is not based on direct evidence. Germination is accompanied by a considerable metabolic activity, and it is not unlikely that the wall of the germ cell is formed during that period.

These considerations lead to the conclusion that the spore coats which have been demonstrated with certainty are two in number. Since the terms *exine* and *intine* as used by various investigators do not indicate the same structures, or indicate the same properties, future use of these terms is bound to lead to confusion.

The literature reviewed above shows that the views on the structure of the protoplasm are as varied for the spore as they are for the vegetative cell. However, the work of the last decade, chiefly that which used the Feulgen technique or a procedure derived from it, has been consistent in demonstrating, within the protoplasm of the spore, what appears as a granule or a rod giving color reactions similar to those of a nucleus. Unfortunately, the significance of these results is reduced by the necessity of hydrolysis and the imperfect specificity of the reactions. There remained the possibility that the bodies observed were not chromatin or, assuming specificity of the reactions, that they were artifacts which did not pre-exist as such in the untreated spores. Only recently (98) was it possible to prove the existence of at least two nuclei in the forespore of *Bacillus mycoides*. These nuclei are at first polar, but later may occupy other positions. A similar picture was observed with the light microscope in spores which used up part or all of their ribonucleic acid but failed to germinate. Such spores stain readily without a previous treatment and show nuclei in polar and other positions. Stained by the Feulgen technique, the spores show what appears to be one to several nuclei within the cytoplasm. Frequently, a nucleus may be extra-cytoplasmic or may even project outside of the spore on a sterigma-like attachment. This was not usually observed in similar material stained without previous hydrolysis, and we believe that it is an artifact caused by hydrolysis in germinating spores, since such spores do not show the uniformly thick, Feulgen-positive rim characteristic of resting spores; the rim is always thinnest in the

neighborhood of the nucleus. Careful observation also shows that what often appears as a single granule or rod is in reality a group or a string of nuclei; chemical treatments accompanied by alternate drying and wetting are known to cause the translocation of intracellular bodies (94).

Of considerable interest is the observation (96) that the forespore and the young endospore of *B. cereus* give a homogeneous Feulgen reaction, whereas the mature spore shows a differentiated nucleus. The records from which this conclusion was drawn are unmistakable, but the conclusion itself is not in harmony with what is now established for *B. mycoides*, and it is doubtful whether the difference should be attributed to a difference between the organisms investigated. It is conceivable that the phenomenon observed is due to an autogamous process which takes place during maturation; more likely, however, it is an artifact of the Feulgen procedure. It is known (89), for instance, that ribonucleic acid would give a positive Feulgen reaction were it not removed from the cell by the hydrolysis with normal HCl, and the homogeneous reaction given by the forespore and the young endospore may mean that the ribonucleic acid is here present in a stable chemical combination not readily broken up by hydrolysis with HCl, and that it is spontaneously liberated during maturation. Indeed, the difficulty with which ribonucleic acid is removed from the young, vegetative cell by hot water and alkalis (98) suggests that it is present in these cells in a stable combination; in many species it is later spontaneously liberated and precipitates out as volutin. This interpretation is further strengthened by the observation (148) that stainable material is more readily removed by hydrolysis from the spore than from the vegetative cell.

Therefore, it may be concluded that the resting endospore consists of a dense cytoplasm containing usually two or more nuclei and a relatively large quantity of ribonucleic acid. This cytoplasm is surrounded by a cytoplasmic membrane and by two coats, of which the inner one is the more rigid, the outer one the more elastic. An idea to the degree of physical toughness of the spore coats may be gained from experiments reported by Curran and Evans (30).

### *Cytology of formation*

The cytological processes involved in the formation of the endospore have been the object of numerous investigations since the early history of modern bacteriology. The considerable literature that has accumulated lacks harmony both in fundamentals and in details. It is needless to emphasize that a real diversity would be most valuable in the taxonomy of sporulating bacteria, but there is more than a mere suspicion that the reported differences are often more characteristic of the investigator than of the organism investigated; and it is possible, by a rigorous critical analysis, to reach the conclusion that the endospore is formed by a series of cytological processes which are fundamentally the same in all spore-forming bacteria.

The reported methods of sporulation have been classified by the author (87, 90) under three headings: *a*, growth of a granule; *b*, aggregation of granules; *c*, condensation of the protoplasm. This classification will also be followed here.

a. *Growth of a granule.* Under this heading we include all methods in which the endospore is said to result from the growth of a granule already present in the cell or of one which appears at the time of sporulation, but not in an especially differentiated part of the protoplasm, whether its growth takes place at the expense of the cytoplasm, of cytoplasmic inclusions, or of both.

According to Cohn (23), when the threads of *B. subtilis* are ready to sporulate, there appear in their homogeneous content highly refringent, small bodies each of which develops into an oblong, or a cylindrical, highly refringent spore with a dark contour. Describing the process in *B. anthracis*, Koch (104) stated that, previous to sporulation, the content of the most vigorously growing threads becomes finely granulated, and there appear at regular intervals, small, shiny granules which, after a few hours, enlarge into the strongly refractive, rounded spores. As observed by De Bary (36) in *B. megatherium*, sporulation is initiated by the appearance of small, round, highly refractive granules arranged along the periphery of the protoplasm. This is followed by the appearance of a small, round, highly refractive body near one of the extremities of the cell; one gains the impression that this body is the result of growth of one of the granules mentioned above. The body then grows perceptibly larger while the protoplasm around it diminishes. In the course of a few hours, it grows into an elongated, cylindrical object which is shown by subsequent behavior to be a spore. Ernst (58, 59) studied the process of sporulation in organisms from boiled hay infusion. The cells contain each from 2 to 6 "sporogenous granules" which stain blue-black with warm, alkaline methylene blue, and violet-black with Delafield's hematoxylin. One of these granules grows, gets differentiated into a coat and a nucleus, and becomes a spore.

Schaudinn (154) studied a marine organism, *Bacillus sporonema*. The vegetative cells of this organism are said to divide by constriction and to be sparingly granulated except in old cells preparing to sporulate. Sporulation is initiated by the appearance of a constriction in the middle of the cell. After 3 to 4 hours there appears in the constricted part of the cell a small, spherical body which enlarges gradually; this body is less refractile than the granules of the cytoplasm and gives the impression of being a vacuole. However, it stains dark with iron hematoxylin and retains the stain after the entire protoplasm has been decolorized. This, Schaudinn calls the *forespore*. As this forespore grows, the granules distributed in the protoplasm become less refractile and gradually disappear, the process proceeding from the poles of the cell toward its middle. No streaming of the granules was observed. This is followed by an alteration in the form of the cell into a spindle-like body consisting of three swollen parts separated by two constrictions, and then by a period of about one hour in which the poles of the spindles grow in opposite sense into long threads, becoming thinner as they elongate. The forespore continues to enlarge, becomes more refractile, and one observes at its surface a sharp, dark line. The mature spore is so refractile that no details can be seen inside of it.

A similar method was also described by Guilliermond (70) for *Bacillus radicosus*, *B. mycoides*, and several other members of the genus *Bacillus* (*B.*

*megatherium*, *B. subtilis*, *B. alvei*, *B. asterosporus*, *B. tumescens*, and *B. limosus*). When the culture is about 24 hours old, the alveolar cytoplasm of the cells contains a large number of small granules situated at the knots of the network. These could be considered chromatic granules or chromidia. Sometimes these granules are more concentrated in the axial region and present the appearance of a spiral chaplet. At the time of sporulation, one observes near one of the poles, a small granule, visible in the living cell and resembling a vacuole. At first, this granule has an irregular contour but later becomes spherical. It has all the characters of a nucleus, staining dark red with safranine and black with iron hematoxylin. The cytoplasm becomes more and more acidophilic. The granule enlarges, assumes an oval shape, surrounds itself with a membrane which, as it becomes denser, opposes the penetration of dyes. At this time, the spore can not be stained by ordinary means; only its membrane stains lightly. The spore surrounds itself by a very stainable, amorphous mass which appears to result from the condensation of the unutilized cytoplasm around the spore. During the development of the spore, the cytoplasm remains granular. Therefore, the spore does not appear to result from the condensation of the cytoplasmic granules but, at most, of a portion of these granules. Indeed, a large part of these granules persists during the formation of the spore. The spore continues to grow at the expense of the portion of the cytoplasm which had not been used up by the forespore.

Although the process described by Guilliermond (70) and that described by Schaudinn (154) for *B. sporonema* are similar in that both assume the growth of a nucleus-like granule, they are distinct in that Guilliermond considers that growth takes place chiefly at the expense of the cytoplasm and, possibly, at the expense of a portion of chromidia lying in the cytoplasm, whereas in the case of *B. sporonema* the nucleus-like granule is said to grow at the expense of more refractile, cytoplasmic granules which completely disappear during the formation of the spore. However, a further statement by Guilliermond that, "dès l'apparition de l'ébauche de la spore, le cytoplasme perd peu à peu ses granules chromatiques" suggests that the two methods may not be fundamentally different.

We also classify here the process observed by Dobell (46) in *Bacillus spirogyra* and *Bacillus lunula*, two organisms found in the large intestine of frogs and toads. In *B. spirogyra*, the vegetative cell is said to contain a spiral filament of chromatin which runs from end to end. "A large individual divides into two in the ordinary way—the chromatin filament being the first to divide. The daughter-cells produced by this division separate and undergo a certain amount of growth, though they never reach the size of the 'parent' cell from which they were formed. Inside the cell the filament is seen to consist of comparatively few turns, and frequently displays a slight knob at one or both ends. One end of the filament now begins to enlarge—apparently at the expense of the rest of the spiral—so that a large nucleus-like mass is formed at one end of the cell. Up to this stage this body—the spore rudiment—stains deep red, like chromatin, with Giemsa's stain. But a little later it changes its reaction, and is stained a

bright blue. This . . . is owing to the formation of the spore membrane. As the membrane hardens the spore gradually stains less and less—until, finally, it refuses to stain at all. . . . It must be noted that a part of the filament persists outside the spore until quite a late stage, and breaks up with the rest of the cell." Occasionally the large, original cell fails to divide completely, "and each of the small daughter individuals had developed spore rudiments whilst still attached to one another."

In *B. lunula*, sporulation takes place as in *B. spyrogyra*, except that about half of the sporangia contain two spores each as a result of failure to divide completely.

Growth from a single granule was recently described by Hoyt (78) in the anaerobic *Clostridium septicum*. "The change from a solid-staining granule to a spore apparently involved a swelling of the granule followed by clearing of its central portion,—the end result being a typical refractile spore with a deeply staining circumference." He adds: "We can not say, however, that this is the only mechanism of endospore-formation in *Cl. septicum*."

b. *Aggregation or fusion of granules.* This includes the methods in which granules, usually of chromatin, visible with the light microscope and originally distributed throughout the cytoplasm migrate to a definite region where they fuse together, are surrounded by a membrane, and become the spore.

Bunge (14) severely criticized Ernst (58, 59) for the sporogenous role he attributed to his granules, since these may be present in nonsporeforming bacteria and be absent in typical spore formers such as *B. anthracis* and *B. megatherium*. According to Bunge, spores are formed from material characterized by its resistance to staining. This material appears in the form of granules in the bacterial protoplasm and grows by confluence and apposition to the size of the spore.

Burchard (15), referring to *B. petroselini*, stated that it sporulates by forming granules which later fuse, a process also observed in *B. subtilis*. Migula (124) described a similar process in *B. asteroides* and considered the fusion of granules as the method by which most bacteria sporulate, although he had previously (123) expressed a similar opinion about growth from a single granule at the expense of cytoplasmic inclusions.

This method, however, receives its chief support from the work of Schaudinn (153) on *Bacillus bütschlii*. When one isolates, in a droplet of intestinal juice of the cockroach, a coarsely granulated rod, one observes in about 30 minutes the appearance of a large, shiny granule in the middle of the cell. As in the case of vegetative division, this granule becomes broader and grows in 20 to 40 minutes into a cross-wall. After 1 to 2 hours, the cross-wall becomes less refractive and disappears completely in about half of an hour. Then one notices motion of the granules in protoplasmic streams. In about a quarter to a half hour, the velocity of the granules increases. The granules at the center of the cell move in an opposite sense to those at the periphery. In about a half to 1½ hours, active streaming subsides and is replaced by an irregular, slow streaming which results, in about a half hour, in the arrangement of the granules in the



middle of the cell into a zigzag band which stretches from pole to pole. Simultaneously, the granules begin to gather at both poles; this marks the beginning of spore formation. Within the next half to 1 hour, the forespore grows at the expense of the band which becomes gradually narrower and shorter. The young forespore is sharply delimited and resembles the alveolar nucleus of many protozoan cells. When it becomes thrice as long as wide, it begins to contract; the contraction causing loss of the alveolar fluid. The granules come closer and finally fuse together. Because of the loss of fluid, the forespore moves from its polar position toward the middle of the cell. It becomes smaller but more highly refractive and more stainable than before. The net-like structure becomes invisible in the living state, and the forespore less and less stainable; its outline becomes sharper and darker.

The light alveoli which during contraction surrounded the forespore with a regular alveolar seam now gradually lose their content, and the substance of the alveolar walls becomes more refractive, forming around the shiny forespore a homogeneous, weakly stainable zone. The outer boundary of this zone condenses into a strongly refractive membrane with a double contour. As this membrane is formed, one observes a thickening of the alveolar cell substance at the proximal poles of both spores. This material serves for the formation of a second coat which surrounds the spore except for a zone at the outer pole where the germ cell will emerge. Flagellar motion stops at the time the forespore becomes surrounded with the first membrane, sometimes sooner. Occasionally, one observes weak motion of a cell with fully ripe spores.

Dobell (46) described a similar process in *Bacillus flexilis*, an organism found in the large intestine of frogs and toads. "A large individual, containing numerous chromatin granules, almost divides itself into two equal daughter-cells. The division is not completed, and subsequently all trace of it disappears. The granules now arrange themselves in the form of an irregular spiral, and at the same time begin to travel to opposite poles and mass themselves together to form the spore-rudiments. Round these is formed a spore membrane, which gradually hardens and so gives rise to the completed resistant spore. The remains of the cell, including a part of the chromatin spiral, perish."

*c. Condensation of the protoplasm.* Under this heading we include those methods in which the endospore is said to be initiated by the sudden appearance, in a previously differentiated part of the protoplasm, of a primordium or of a forespore with dimensions exceeding, equalling, or slightly below those of the mature spore. Use of the term condensation was criticized by Lewis (114) who, referring to the spore primordium, wrote: "The contents continue to increase in density after the membrane has formed. . . . It seems improper, therefore, to speak of the primordium as a structure formed by condensation of material. The greater density is not due to a mere aggregation of material formed elsewhere but to a greater capacity to form new substance." We are unable to share Lewis' view because condensation simply means an increase in density regardless of the means by which it is accomplished. The mechanism of increase in density will be discussed below.

This method was first described by Brefeld (10) who observed that the formation of endospore in *B. subtilis* begins by the appearance, usually in the middle of the cell, of a dark shadow which becomes more distinct as the surrounding part of the cell becomes lighter, as if the substance of the cell gathered in one place. This place becomes the spore when the process of gathering of the cell substance ends. The spore appears as a dark, strongly refractive nodule within the mat contour of the cell. Accordingly, there is increase in refringence but no increase in size during the development of the spore.

Studying the process of sporulation in a number of bacteria, including *B. subtilis*, *B. anthracis*, *B. megatherium* and *Clostridium butyricum*, Zopf (175) arrived at the conclusion that it consists first of a contraction of the cell content into a smaller space where the mass becomes denser, round, and surrounds itself with a firm, smooth, unstainable membrane probably consisting of two layers. Sporulation by contraction was also described by Klein (82) in the following members of the genus *Bacillus*: *B. solmsii*, *B. de Baryanus*, *B. peroniella*, *B. macrosporus*, and *B. limosus*. The cell swells where the spore is to be formed. The plasma of the swollen part assumes a greenish tone, contracts away from the cell wall, and assumes the bean-like shape of the endospore. Only later does the endospore acquire its shiny, bluish-green color. Continued nutrition of the spore during contraction is not considered likely by the author, since before sporulation the cell is divided into a sporeforming part and a nonsporeforming part. On the other hand, nutrition of the contracted spore is considered probable. The author is uncertain of the stage in which the membrane is formed. He observed no turbidity of the protoplasm during sporulation; the sporangium remains hyaline and alive, since it remains actively motile.

Peters (134), describing sporulation in a *Bacillus E* isolated from sour dough, states that at the time of sporulation there appear in the cytoplasm a large number of fine granules. This is followed by the appearance, near one end of the cell, of a protoplasmic bridge, more refractive than other cell parts (with the exception of the granules). In this bridge appears the spore in the same dimensions as the mature spore, but with a poorly delimited outline. Further development consists of an increase in the refringence and the formation of a membrane. Even as the spore makes its appearance, one observes among the fine granules, at the end of the sporangium opposite to the one containing the spore, one or several larger granules which increase in refringence with the spore; finally, one may see in a barely visible rod the strongly refractive, oval spore with one or several strongly refractive, spherical granules. These granules, however, are not viable.

Frenzel (63) described sporulation in green bacteria found in the intestines of tadpoles, in which he believed to demonstrate a central body. A "spore nucleus," resembling what other investigators call forespore, suddenly appears inside of the central body and develops into a spore. Sometimes a "spore nucleus" may divide by constriction; this results in the formation of disporic sporangia. Frenzel describes a highly refractive halo or a glassy capsule around the spores. It must be stated at this point that the "green bacteria" studied by Frenzel are

probably not true bacteria but blue-green algae similar to the species of *Oscillospira* studied by Delaporte (38, 40) which are also present in the intestines of the tadpole (see below).

Meyer (120, 121) investigated the process of sporulation in *B. asterosporus*, *B. tumescens*, *B. subtilis*, and *Granulobacter*. In the potential sporangium, granules of inclusions appear and are uniformly distributed throughout the cell. Spore formation is initiated by the appearance of a spore vacuole which is at first much less refractive than the surrounding cytoplasm. This vacuole expands, pushing away the trophic plasma and the inclusions until the inclusion-free part of the cell is almost as long as the complete spore. At the same time, the spore vacuole becomes more and more refringent than the trophic cytoplasm in which lie the granules. At this stage, the sporangium is divided into two parts, the fertile part and the trophic part. The fertile part consists of the spore vacuole and the surrounding cytoplasm. The fertile part stains homogeneously with fuchsin, although the spore vacuole contains cell sap vacuoles. A nucleus can be seen enclosed in the spore vacuole. Soon this vacuole becomes more clearly separated from the surrounding fertile cytoplasm. This is the forespore; it becomes more refractile and forms a membrane which finally becomes pointed at the ends.

Preisz (142), studying *B. anthracis* and *Clostridium tetani*, divides the process of sporulation into three stages: 1, formation of the "Sporeanlage" or spore primordium; 2, formation of the forespore; 3, formation of the spore.

Formation of the spore *primordium* is initiated by the appearance, near one pole, of a sickle-like, polar cap which stains strongly with basic fuchsin. This structure seems to be formed by the outermost layer of the protoplasm. At this stage, the cell usually contains several light, unstained granules, the acid-fast bodies. Near the cap of chromatic substance one often observes a round, sometimes elongated, strongly stained nucleus. The spore primordium and the nucleus are separated from the sterile part of the cell by a chromatic cross plate which gives it a biconvex, plano-convex, or concavo-convex shape. This is followed by the appearance, at the place of the cross diaphragm, of a thin, sharp line. At this stage, the primordium has the same thickness as the cell but is  $\frac{1}{3}$  to  $\frac{1}{2}$  as long. It now appears in the fertile part of the cell as a slightly flattened or regularly spherical structure which often stains more deeply than the rest of the cell, and in which no chromatic substance can be demonstrated other than a darkly staining granule lying at the edge and often appearing to be in the contour line. The primordium grows in length until this exceeds the length of the mature spore and, as it grows, it separates from the wall of the mother cell and moves toward the center. At this stage, its contour is clear-cut and as fine as that of the mother cell, but stains with aqueous fuchsin stronger than the latter; its content, with the exception of the less frequently observed nucleus, is homogeneous and stains more deeply than the surrounding cell plasma. On the other hand, it stains with dilute methylene blue solutions as do the germinating spore and the germ cell, i.e., the outermost layer and the center stain more deeply than the rest of its content. However, the dark center is not identical with the fuchsin-stained granule.

When the primordium completes its growth, it becomes the *forespore*. The forespore is often surrounded by a light, unstained meniscus which, Preisz believes, is due to shrinkage of the forespore protoplasm.

The change from forespore to *spore* consists of the differentiation of the content of the forespore. The nucleus-like body which stains strongly with dilute fuchsin is no more visible; it appears as if it dissolved in the content of the forespore. In the center appears an elongated, oval structure the width of which is one-third to two-thirds that of the forespore. This structure belongs to the final spore and is called the young spore; around it, the outermost structure of the forespore appears as a halo. At first, the young spore stains more deeply than its halo; later, however, it loses its stainability and gradually becomes more refringent; simultaneously, its outline becomes more clear-cut and, after maturing of the spore, becomes yellowish or brownish. The halo shrinks into the spore coat. As a rule, the shrinkage is not uniform, and one sees at one, or at each, pole of the spore a crescent-like cap. The work of Preisz is reported in detail chiefly because of the influence it has exerted on subsequent students of the endospore.

Georgevitch (65) described spore formation in two strains of *Bacillus thermophilus* as follows: A period of vegetative growth is followed by the appearance of Bunge's granules in the cytoplasm; then a cross-septum is formed near one of the poles, thus dividing the cell into two unequal parts; sometimes this septum is formed within the curved part of the cell end. A certain number of granules are now seen in the cytoplasm, lying against the lateral wall. Between these granules one sees certain ones which are larger and which stain more deeply; two of these granules lie near a pole, the rest near the middle of the cell. These granules become larger by condensation of chromatic material and finally unite to form a cross-septum which divides the cell into two unequal parts. At first this septum is plane, but later becomes convex. The edge of this septum adjacent to the lateral wall of the cell grows lengthwise, and it is probable that this growth is helped by the granules which lie against the lateral wall. Continuation of this growth accompanied by deposition of chromatic material results in the formation of a vesicle, the forespore. The two chromatic granules present in the forespore disappear and are probably absorbed by the cytoplasm. The significance of these granules is not understood. The forespore then grows lengthwise and moves toward the sterile pole. At first, the wall of the forespore is thick and its outline not sharp; later, however, it becomes thin and sharply delimited. At this stage, the forespore has attained its normal size and form, and chromatic granules arranged along a curved line are formed in its lightly staining protoplasm.

Petersen (135) observed that the spores of *B. mycoides* are always formed around a nucleus lying most often at one of the poles. At the beginning, the region surrounding the nucleus is rich in a volutin-like material, but this is later dissolved and replaced by a bigger and more stainable body which does not give the volutin reaction.

Christian (20) described spore formation, in an organism which causes spoilage

of commercially heated milk, as follows: "After thirty-six to forty-eight hours' incubation a characteristic stage may be observed in which an unstained area is lying towards one end of each cell, cutting off and isolating a definite terminal part of the cell contents. This terminal portion is the potential spore and its position is fixed at this stage of the development. The remainder of the cell contents, meanwhile, have shown definite signs of contraction and finally condense to form a second body, which we have called the 'secondary granule' in order to distinguish it from the terminal or 'spore granule'. . . . At a slightly later stage the position of the 'secondary granule' is seen to vary somewhat in different cells. In a definite proportion of the cases, however, it is oval or lens-shaped and lies closely adjacent to the 'terminal granule'. Whether there is any actual contact between them has not yet been established, but it is of interest to note that it is in such cells that the first indication of a spore wall is visible, and that as soon as this wall begins to form, the staining and refractive properties of the 'spore granule' become those of a true spore. The fate of the 'secondary granule' is uncertain, but it would seem from careful observations that, having played some part in the development of the spore, it disperses throughout the body of the mother cell, which then resumes its ordinary staining capacity.

The first incontrovertible description of sporulation by condensation of the protoplasm was made by Bayne-Jones and Petrilli (5) who used cinematography to record their observations on *B. megatherium*: "As the culture becomes older, round granules of various sizes become very numerous in the cells. . . . There is no evidence of a streaming of granules to indicate a flowing of the protoplasm. In some cells, granules half as large as a mature spore appear and become surrounded by a clear zone. Two of these large granules may appear in a single cell. They have no connection with the formation of the spore, since they remain as granules in the cell after the development of the spore. . . . When multiplication has stopped in most of the cells and when growth is continuing only slowly in others, the ends of the rods begin to lose their granules . . . by displacement of the granules from that region and not by a liquefaction or fusion of the granules previously visible there, or by the growth of a special granule in this area. The clear area at the end of the cell fluctuates in size and gradually comes to occupy a cylindrical volume equal to approximately one-quarter of the volume of the cell. The material in this cleared area appears to be denser than the rest of the protoplasm of the cell. Granules in violent motion in the remainder of the cell strike against the edge of this cleared area but never penetrate it. This large cleared area is the region in which the spore appears."

"The clear area at the end of the cell keeps a roughly cylindrical form for three to four hours. Its density increases gradually and it gradually assumes an almost spherical shape. . . . The rest of the process is finished within the next thirty minutes. A stage which we have called the 'prespore' appears as a dense refractile region in this cleared area at the end of the cell. Its volume is smaller than that of the original clear area but often twice that of the final spore. Within six to twelve minutes, this nearly spherical 'prespore' contracts to an elongated ellipsoid and has a density as judged by its refractile property, as great as that of

the mature spore. . . . Within six minutes or less, this elongated ellipsoidal spore contracts to the somewhat shorter, but apparently no broader ellipsoidal spore, characteristic of this organism."

"During these terminal stages of spore-formation and after the development of the spore, the movement of the granules becomes less and less in the remaining protoplasm of the cell. Nevertheless, some of the granules may now pass into the area near the end of the cell, along the side of the spore, into a region that was too dense to admit them before contraction of the sporogenous area. . . . We have not seen the development of a membrane around the material of the spore."

"The volumes of the cells containing endospores varied from 6.9 to 12.5 cubic micra. The volumes of the contained spores were 0.55 to 0.97 cubic micron, with an average of 0.84 cubic micron. The average ratio of spore volume to cell volume was 0.09. But this average obscures some considerable differences in this ratio, which varied from 0.06 to 0.13." Here belongs also the method described by Badian (3) for *B. subtilis* and *B. mycoides*, and later (4) for *B. megatherium*. Details of the cytological processes involved will be reported and discussed in a later section.

The observations of Lewis (114) on *B. mycoides* are as follows: "The first indication of spore formation to be noted in the living unstained cells is the clear polar spore primordium. . . . The granules are localized in the nonfertile segment of the cell, and there seems to be no evidence that they play any direct part in formation of the primordium. There is no refractile sporogenous granule present in the young primordium and no evidence of shrinkage or plasmolysis of the cell contents."

"When the material of the proper age is examined in dilute fuchsin solution, the primordium is clearly differentiated by its greater staining capacity. . . . The cell has been differentiated into two distinct areas, a fertile portion destined to further development as a reproductive structure and a sterile portion containing reserve nutritive material, incapable of further divisions and destined to die. . . . There is no conclusive evidence that the deeper staining capacity of the spore primordium is due to a difference of chromatic material. . . . The material, except for a single deeply stained granule which is not always visible, has every appearance of uniformly stained cytoplasm. . . . The earliest stages of membrane formation are similar to that described by Preisz, (1904), for *B. anthracis*. The membrane extends iris-like from the extreme end of the rod, enclosing all but a thin layer of cytoplasm lying against the original cell wall. . . . The contents continue to increase in density after the membrane has formed."

"Just preceding differentiation to form the vospore the primordium has become quite dense and stains deeply with dilute fuchsin or gentian violet solution. . . . Differentiation within the primordium appears to be a true process of condensation. In unstained living material, a central refractive body forms while the periphery appears as a clear hyaline zone. When stained vitally with dilute gentian violet, the central body while young is more deeply stained than the zone surrounding it." These two differentiated zones are called endosporium and exosporium, respectively.

At the time of the differentiation, the endosporium is about  $\frac{2}{3}$  the diameter of the forespore and resembles in size and shape the ripe spore. The exosporium stains with aqueous dyes more deeply than the cell protoplasm and is gram-positive. The forespore invariably moves from its polar position toward the middle of the cell. The exosporium becomes elongated forming a cylindrical body which is longer than the endosporium but only slightly greater in diameter. It retains its staining capacity and appears in the form of large, deeply staining hemispherical bodies at the ends of the endosporium. The spore wall is formed between the endosporium and the exosporium and not, as Preisz believed, by shrinkage of the exosporic layer. The granules diminish in number during the ripening of the spore and are generally absent in old sporangia, either because they are used up in the process of ripening or by the mother cell in its own metabolic processes.

Delaporte (38-40) studied endospore formation in the cyanophyceae *Oscillospira guilliermondi* and *Oscillospira batrachorum* as well as in *Spirillum praeclarum* and other large bacteria found in the intestines of the tadpole. In *Oscillospira guilliermondi*, the spore is formed by long cells inside of the central body. The young spore stains intensely with Lugol's solution and, for a time, remains surrounded by a thin layer of glycogen which later disappears. It stains intensely with iron-hematoxylin, methylene blue and safranin, and is surrounded by an unstained, thin layer; this layer is itself surrounded by a chromatic, irregular pellicle which is a residue of the central body, and which is subsequently absorbed by the spore. As the spore elongates, it loses its chromaticity. Before maturity, it often shows small, internal, siderophilic granules arranged like a chaplet; these grains give a positive Feulgen reaction even in the mature, unstainable spore.

In *Spirillum praeclarum*, the vegetative cell has, according to Delaporte, a long, chromatin-containing axial filament. Before spore formation, one observes a difference of chromaticity between the two halves of the cell; in one-half the axial filament contains more chromatin, and the cytoplasm stains more strongly with erythrosin, than in the other. The forespore is formed in the more chromatic half. The chromatic substance gathers in the center of the oval forespore in the form of a sinuous filament or a crown. Around the forespore, there is accumulation of siderophilic substances which are later assimilated by the spore together with the rest of the axial filament. In contrast with the endospores of other bacteria, the mature spore of *S. praeclarum* remains strongly stainable with erythrosin. Spore formation in the other large bacteria found in the intestines of the tadpole takes place in a generally similar manner. This method differs from the one described by Dobell (46), for *Bacillus spirogyra* and *B. lunula*, in that the chromatic substance does not constitute the entire forespore as it is said to do in Dobell's organisms.

Roberts (145) described sporulation in *Clostridium pasteurianum*. The spore is formed in a granule-free region at one pole of the cell. This region rapidly enlarges until it occupies about half of the cell volume. It stains more deeply with basic dyes than the rest of the cell, and appears clear when mounted

in iodine in contrast to the reddish brown color assumed by the granulose-containing, sterile portion of the cell. This suggests that the material from which the spore is formed aggregates in the sporogenous region by the migration of certain basophilic cell constituents. Observations on factors influencing spore formation suggest that initiation of the process of sporulation may depend on a lowering of protoplasmic viscosity.

The process of sporulation in *Bacillus cereus* was recently studied by Knaysi (97). The organism was grown in microcultures more suitable than those used by previous investigators. In addition to intensive, direct, observations, serial photographs were made at regular intervals. The process was divided into three stages, the preparatory stage, the forespore stage, and the maturation stage. The fundamental process during the preparatory stage is the differentiation of the cell into a dense, fertile part and a less dense, sterile part. Although sporulation can take place in inclusion-free cells (94), lipoprotein inclusions are usually present and move, during this stage, to the sterile part of the cell.

"The first evidence of a forespore is the sudden appearance, in the hyalin, inclusion-free part of the sporangium, of a faint, elliptical contour which almost touches the cytoplasmic membrane of the sporangium. . . . This contour line represents the intersection of a thin, ellipsoidal envelope with a plane containing the long axis of that envelope and normal to the axis of the microscope. This envelope consists of material similar in composition to that of the cytoplasmic membrane. . . . It appears *in situ*. At first, the material within and without the envelope has the same refractive index . . . and the outline of the envelope is very difficult to see without staining; but the refractive index within the envelope rises rapidly, so that, usually within 10 minutes, the forespore becomes a highly refringent body. . . . There is not the slightest evidence for the contention of Meyer (120-122) that the forespore contains a relatively huge vacuole."

During maturation, "the forespore undergoes changes in form and size. It decreases in width with little or no change in length, thus assuming a more slender form and smaller volume . . . ; it becomes the endospore." After a period of a few hours, "the inclusions begin to disintegrate. . . but the sporangium often breaks up before this disintegration is complete, and the inclusions, or their residue, are liberated into the medium." The ratio between volume of the spore and volume of the sporangium varied between 0.11 and 0.14; that between volume of the endospore and volume of the forespore varied between 0.41 and 0.82. No study of the development of the spore coat was made.

Although the process of maturation still requires further investigation, considerable elucidation of the earlier stages resulted from recent studies by Knaysi and Baker (98) of *Bacillus mycoides* with the electron microscope. When thoroughly washed spores are heavily inoculated into a medium containing glucose and sodium acetate but no nitrogen source, they germinate and the organism is able to complete its life cycle. The transparency of the cells makes observation of the internal structure and its behavior easy, and one can see forespores at different stages of development. A forespore seems to be formed



in the following manner: The nuclei of the cell which is ready to sporulate, 2 to 6 in number, gather into two groups of 1 to 3 nuclei each. The distance between the distal ends of the groups is equal to the length of a spore. One then observes an area of semi-elliptical outline, and denser than the cytoplasm of the mother cell, grow from one group toward the other; the two areas finally merge into one and, together with the nuclei now occupying polar positions, form the forespore. The forespore gradually increases in density, and some of the nuclei may now occupy other positions in the forespore. The details of transition from forespore to mature spore were not observed. Knaysi *et al.* (99) did not observe, in *B. mycoides*, a difference in opacity to electrons between the cytoplasm surrounding the spore and that in the "sterile part" of the sporangium.

*Discussion.* A study of the literature reviewed above shows how difficult it is to classify the reported methods of endospore formation. Only by overlooking details and defining the methods in the manner we did has it been possible to find some order in what appeared to be a chaos. In certain cases, mechanical adherence to definition leads us to classify in one method what we formerly (87, 90) classified in another. For instance, spore formation in *B. sporonema* (154) is said to take place by growth of a body at the expense of granules present in the cytoplasm; formerly, we had interpreted this as condensation; in this review the method is classified under growth of a single granule. The departure is justified by the possibility that the material of the cytoplasmic granules may not have caused growth by direct opposition of its dissolved molecules, but these molecules may have been used up to furnish energy and some material for growth. The same applies to the method described by Guilliermond (70).

The question now is whether or not there are three different ways by which bacteria may form endospores. An affirmative answer would, indeed, be taxonomically desirable. Unfortunately, no categorical answer can yet be given to this question, since a categorical answer requires reinvestigation of many sporeforming bacteria. However, critical analysis of the literature and recent knowledge enable us to anticipate a negative answer, *i.e.*, that bacteria do not form endospores in three different ways. It may be recalled that the same organism, *e.g.*, *B. subtilis*, *B. megatherium*, *B. anthracis*, or *B. mycoides* are said to sporulate in one way by one author and in an entirely different way by another. Could this be due to differences in strains or investigators? It does not appear likely that Meyer (120) and Migula (124) investigated two different strains of *B. asteroides*. In certain cases, (78) however, it is possible to show that what has been considered a granule may well have been a forespore in the usual sense. Endospore formation takes place when growth in the culture has practically ceased. Its suddenness, its sensitivity to the environment, and the usual presence of inclusions in cells ready to sporulate make accurate observation extremely difficult. Only by frequent observation and exact recording of changes taking place in the same cells can one be certain of the sequence of events. Photographic recording is not a luxury but a necessity. Whenever the process of sporulation was thus investigated, it was found to take place by the formation of a forespore which appears suddenly with dimensions greater than those of the completed spore.

The work of Knaysi and Baker (98) in which the role of the nuclei in the formation of the forespore was demonstrated suggests that there is no sharp line of distinction between what we called sporulation by condensation of the protoplasm and sporulation by the aggregation of granules. The difference may be in the number of nuclei which take part in the formation of the forespore.

### *Germination of the endospore*

Describing spore germination in *B. subtilis*, Cohn (23) stated that the spore swells somewhat and pushes, *at one end*, a short germ tube. The strongly refractive body of the spore soon disappears; at this stage the germ tube resembles a short rod which begins to move and divides, elongating into a thread. Koch (104) described the spore germination of *B. anthracis* and, with Cohn's help, illustrated the life history of that organism. Under high magnification, the spore appears oval and imbedded in a spherical, transparent mass in the form of a narrow ring. This mass loses its spherical shape and elongates in the direction of the long axis. The spore (meaning the shiny body) remains at the pole of the now cylindrical mass; at the same time it begins to lose its refractivity, becomes smaller, breaks up into several parts, and finally disappears.

During the following thirty-two years, spore germination was the object of a number of investigations concerned chiefly with changes in gross morphology, behavior of the spore coat, and application to taxonomy. It was found that germination always begins by swelling of the spore and decrease in refringence. The extent of this change varies with the organism. At this stage, the permeability of the spore coat becomes similar to that of the vegetative cell (69, 70). In the majority of sporeforming bacteria, this stage is followed by bursting of the spore coat and emergence of the germ cell. In other bacteria, no coat is shed; it is either absorbed or gelatinized and dissolved; in such organisms sometimes a fragment of the coat remains tightly adherent to the end of the germ cell. In the group that sheds a coat, the coat may burst at the pole (polar germination) or laterally (usually called equatorial germination). The coat may be shed immediately or may remain attached to the end of the vegetative cell for many generations.

Variation in the mode of germination was observed. Among organisms with polar germination, there are some in which bipolar germination may be frequent (83, 168, 67), or may predominate (15). Lateral variation was observed in organisms said to have equatorial germination (69). In *Bacillus loxosus* (15), the coat is regularly punctured between the pole and the equator. Polar and lateral germinations were observed in *Bacillus brassicae* (140) and *B. subtilis* (67). According to Migula (125), shedding or absorption of the coat in *B. anthracis* depend on the medium.

The value of the mode of germination in the taxonomy of the sporeforming bacteria has been a subject of debate. Burchard (15) exaggerated its constancy and considered it the best taxonomic criterion; Caspari (19) found little constancy in the organisms studied by Burchard; a more reasonable attitude was taken by Gottheil (67) who stated: "My investigations have shown that the mode of germination is of value in the identification of a bacterium, provided all

the modes exhibited by a given species are considered." (Translation by the reviewer.) The modes of germination observed by Gottheil in the various species he studied were classified as follows: 1, polar; 2, bipolar; 3a, lateral, equatorial, with unilateral splitting of the coat and a short germ cell; 3b, lateral, equatorial, with complete splitting of the coat, the two halves of the coat remain as caps around the ends of the germ cell; 3c, lateral, equatorial, with unilateral splitting of the coat, fast growth of the germ cell inside the stretched coat, and long germ cells of comma-like form.

The following is a brief account of the modes of germination of most of the organisms studied during that period:

*Polar* germination was described in *Clostridium butyricum* and *C. polymyxa* (*Bacillus polymyxus*) (141); in *B. anthracis* (141, 69); in *Aslasia asterospora* (*Bacillus asterosporus*) (120); in *Bacillus ruminatus*, *B. ellenbachensis*, *B. pumilus*, and *B. fusiformis* (67); in *B. mycoides* (67, 70) and only in certain strains of the same organism (76); in *B. bütschlii* (153). *Bipolar* germination was observed occasionally in *B. sessilis* (83) and *B. ramosus* (168), and predominantly in *B. bipolaris* (15).

*Lateral* germination was described in *B. subtilis* (141, 10, 36, 69); in *B. megatherium* (36, 69); in *B. inflatus* and *B. ventriculus* (103); in *B. tumescens* (103, 67); in *B. mycoides* (69), and only in certain strains of the same organism (76); in *B. loxosus*, between pole and equator (15); in *B. sporonema* (154).

*Polar* and *lateral* germinations were observed in *B. brassicae* (140); in *B. carotarum*, *B. cohaerens*, *B. petasites*, *B. simplex*, and *B. subtilis* (67).

Germination *without shedding a coat* was described in *B. anthracis* (36); in *B. carotarum* (103); in *B. leptosporus* (83).

The names of the species used in this summary are those used by the respective authors regardless of possible synonymy (see Gottheil, 67). Details and discussions should be sought in the original literature.

During the last four decades, spore germination was not always carried out to study changes in gross morphology or with the purpose of application to taxonomy, but often as a means to study internal structure.

Preisz (143) described the process in various sporeforming bacteria as follows. When a spore is placed in a medium where it can germinate, the first sign of its viability is the presence of a nucleus at the equatorial side of the shiny, reserve body. The nucleus stains deeply. The round, or elongated, nucleus sometimes projects from the contour of the spore as if it were adhering to it from the outside. Inside of the apparently unchanged spore coat, one then observes a darkly stained contour in the middle of which is the shiny body. In a later stage, the nucleus swells at the periphery of the spore, and may reach one-third of the thickness of the spore. Sometimes one finds on either side of the central, shiny body a darkly stained, little nucleus. Throughout these changes, the coat appears to play a passive role; it becomes less visible and begins to take up more dyestuff than the coat of the resting spore. Upon further development, the central, shiny body disappears, and the entire spore body changes into a chromophilic protoplasm, and the nucleus becomes invisible. This is followed by

emergence of the germ cell and shedding of the coat. The germ cell stains faintly and contains one nucleus or, sometimes, two nuclei. The wall of the germ cell appears to be formed during germination. Other interesting details should be sought in the original paper.

Allen, Appleby, and Wolf (1) observed two types of spore germination in an aerobic organism isolated from grass. In both types the germ cell emerges from the pole of the spore. In type A, the spore "loses its refractivity, enlarges to the dimensions of a vegetative cell and divides by transverse fission. It stains feebly and uniformly and shows no internal structure. After several divisions have occurred the presence of refractive granules is apparent." This description conveys the impression of absorption of the coat rather than of polar germination. In type B, when the germ cell emerges from the spore, it "contains a nuclear structure which undergoes division. A central, transverse band of chromatin is resolved into a cross structure, the two halves of which are pulled apart and are soon transformed into minute, separate, rod-shaped structures. Fission of the cell results in two cells each containing one of those rods. The latter then disappears, giving rise to cells which at first stain evenly and rather deeply, but after a few cell divisions they lose their staining capacity and acquire refractile granules."

The mode of germination in various members of the genus *Bacillus* was studied by Lamanna (106) and was correlated with other characteristics, *e. g.*, spore antigen, physiological behavior, and, to a certain extent, heat resistance; the latter relation was previously found by Knaysi (87). Lamanna states: "When the methods of spore germination are clearly defined and separated they have taxonomic value. Spore germination of a given type is constant for any one culture and species." He classifies the modes of germination as follows:

"I. Shedding of spore coat: small-celled species

A. Equatorial.

Not splitting along the transverse axis: *Bacillus subtilis*

Splitting along the transverse axis: *Bacillus vulgatus*.

B. Polar: *Bacillus agri*

C. Comma shaped expansion: *Bacillus mesentericus*.

II. Absorption of the spore coat: large-celled species."

In this classification *B. subtilis* is the strain of Ford and Lawrence recently named *Bacillus licheniformis* (66); *B. vulgatus* is the one known as the Marburg strain of *B. subtilis*.

Spore germination by shedding of a coat is said to have three characteristics: the spore swells only to about the double of its original volume, it does not lose all of its refractivity, and the coat remains visible for a long time after it is shed off. In the first type of equatorial germination, the germ cell forces its way through the coat in a perpendicular or oblique direction to the major axis of the spore. In the second type, the coat is split such as an egg with the two halves of the split shell remaining attached. Germination by absorption of the coat is defined as one in which the spore swells to three or more times its original volume, and its refractivity is reduced to about that of the vegetative cell; the true spore

coat disappears after the second division of the germ cell, although a thin capsule may persist. When such a capsule is visible, its position indicates polar or equatorial emergence of the germ cell; both may be observed in the same culture, but one usually predominates. The capsule, which may be a part of the spore coat, is distinguished from the latter by a low refractivity which approaches that of the germ-cell wall. In a later work, Lamanna (109) observed, in the germinating spores of *B. subtilis* (*B. licheniformis*), gram-positive bodies assuming forms which suggest division. Present knowledge of the structure of the sporeformers permits considering these bodies as nuclei.

Knaysi and Gunsalus (101) studied spore germination in the Marburg strain (C<sub>4</sub>) and in the Lawrence and Ford strain (S<sub>8</sub>) of *B. subtilis*. They stated: "We have reinvestigated the problem, not only in stock cultures of both strains, but also with a number of their variants, and have arrived at the conclusion that strictly equatorial germination in both organisms is far from being the rule. On the contrary, in the majority of the variants most of the germ cells emerge at loci intermediate between the pole and the equator or, not infrequently, at the pole. The proportion of intermediate, equatorial, or polar germination varies with the dissociant; whether it is constant for a given dissociant, we are not able to say. As to the splitting of the exine, we were unable to observe it in S<sub>8</sub> or in any of its variants; on the contrary, this phenomenon often predominates in C<sub>4</sub> and its variants; sometimes less than half of the spores show splitting; in the majority of the rest the exine is bent to a variable degree without splitting; in a small proportion, one usually observes neither bending nor splitting. Following the loss of the germ cell, exines originally split or bent generally reassume the normal form of the spore, indicating a high degree of elasticity." Regarding the taxonomic value of spore germination, Knaysi and Gunsalus wrote: "We . . . are convinced that, as knowledge of that process becomes more adequate, it will become more and more valuable, at least as a group criterion within the genus. . . . In using this and other criteria, however, it must be remembered that the variability of a characteristic may be just as significant as its stability."

Staining with Giemsa's solution after hydrolysis with warm, normal HCl, Robinow (148) observed the following changes in the germinating spores of *B. mycoides*. "Shortly after the spores have been covered with warm nutrient broth, the nucleus actively enters, or is engulfed by the cytoplasm where its outlines become very indistinct. . . . This event is accompanied by a great increase in the (basophilic) affinity of the cytoplasm for the blue component of Giemsa's stain. The differentiation of the cytoplasm into stainable core and glassy outer layer is lost and the cytoplasm now stains blue all over. At this stage it may be difficult to trace the nucleus in spores where the cytoplasm is particularly deeply stained, but I no longer believe it disappears altogether."

"In the course of the next 5 or 10 minutes there is a gradual decrease in the amount of stain absorbed by the cytoplasm, and distinct chromatinic structures reappear. . . . At first, the chromatinic structures are very delicate and look like a somewhat distorted C, S, or V; symmetrical 8-shaped configurations and elongated rings are also common. . . . Gradually all these configurations con-

tract into a thin, knotted string of chromatinic matter in the center of the cell which further contraction (and growth?) turns into a deeply stained, compact, polygonal body. . . . Towards the end of the first hour of incubation this body is found in various stages of division into what appears to be two pairs of chromosomes. . . ."

Germination was recently studied in *B. mycoides*, strain C<sub>2</sub>, by Knaysi *et al.* (99), using the high-voltage electron microscope. "The process is initiated by a slight decrease in opacity and by unilateral swelling. Bilateral swelling is occasionally observed. This is followed by cracking of the inner coat and subsequent tearing of the elastic, outer one. One end of the germ cell slips out of the split and bent coat. Sometimes the two halves of the coat are completely severed and remain as caps around the ends of the germ cell. No internal structure was observed in the germinating spore or in the germ cell. The wall of the germ cell was not visible in the resting spore; but it can be seen as soon as the spore coats are split and may have been formed during germination.

In a more recent study with the electron microscope, Knaysi and Hillier (102) observed shedding of a single, thin coat in a strain of *B. megatherium* heretofore believed to absorb its coat. This coat is usually split between the pole and the equator, and quickly disintegrates leaving a matrix consisting of long, parallel, beaded molecules.

*Discussion.* A study of the literature reviewed above shows a general agreement about the changes in the appearance of the spore during germination. These changes, swelling and reduction in refractivity, have been usually attributed to absorption of water. In the light of present knowledge, however, this explanation becomes inadequate. The resting spore does not stain with dilute, aqueous solutions of basic dyes (69); only the spore coat stains lightly (70). This unstainability has been attributed to the impermeability of the coat; on the other hand, Grethe (69) found it difficult to see why a coat permeable to nutrients is impermeable to dyes in aqueous solutions. It must be remembered, however, that the spore protoplasm stains intensely when placed in a solution of a basic dye for long periods of time or when heat is applied and, once stained, it is not easily decolorized. This is the basis of all spore-staining procedures, and points to the impermeability of the coat as the cause of "unstainability" of the spore. The swelling and decrease in refractivity observed in the early stages of germination are accompanied by an increase in permeability and reduction in resistance to deleterious agents; the spore becomes readily stainable and acquires characteristics similar to those of the vegetative cell, although the properties of its surface, as determined by electrophoresis (50) remain typical of the spore until the coat is shed or otherwise disappeared. The mechanism of change in permeability is not entirely clear, but it may be shown that the change is not due merely to swelling of the spore coat. Meyer (120) expressed the opinion that it is not due solely to stretching but also to chemical elaboration of the coat. The recent observations of Knaysi (92) show that the endospores of *B. mycoides* germinate in solutions of a utilizable source of energy such as glucose. Under these conditions there is no evidence of swelling of the coat; on the contrary, in spores

which germinate very slowly, the coat appears thinner and often disappears without being shed. Recent work with the electron microscope (99, 102) gave no evidence for an increase in the thickness of the spore coats during germination. Indeed, it is difficult to understand why structures already bathed in water, and somewhat permeable both to water and to glucose, should require available food in order to swell. In the case of the spore protoplasm, the evidence for an increase in volume chiefly through physiological activity is clear. A strong fermentation of glucose in a suspension of spores most of which show little or no swelling, and the gradual disappearance of relatively considerable quantities of ribonucleic acid during the early stages of germination have been reported (92, 98). The protoplasm of the spore increases in volume by the normal processes of growth until the spore coat bursts at its weakest part. In the case of *B. bütschlii*, the weakest part is a terminal spot marked, in the resting spore, by a local absence of the inner coat. In strain C<sub>2</sub> of *B. mycoides* which germinates equatorially, the middle part of the coats appears to be the thinnest; this initial difference is magnified by physiological action indicated in electron micrographs by transparent lateral spots of variable dimensions. It is not yet possible to state why this physiological action affects lateral areas of the coats in organisms with lateral germination.

The observation (102) that a strain heretofore considered to absorb its coat actually sheds a single, thin, perishable coat may be found true for other absorbing strains. Whether or not a second inner coat was present in the resting spore and utilized during germination can not be decided.

This discussion leads to the consideration of another phase of spore germination, *i.e.*, its value as a taxonomic criterion. The reviewer is thoroughly familiar with only a few of the common sporeforming bacteria which he has observed, or with which he experimented, over periods of years. Of these, *B. megatherium* always "absorbed" its coats, *i.e.*, it shed a single, thin, perishable coat not easily seen with the light microscope. *B. cereus*, strain C<sub>3</sub>, always germinated terminally, sometimes within its persistent sporangium (87). In *B. subtilis* (Marburg strain) lateral germination is the rule, although in 2 or 3 per cent of the spores germination may be terminal; the coats may be either punctured or split. *B. licheniformis* never splits the coats, but exhibits similar variability to *B. subtilis* in the locus where the germ cell emerges. This is qualitatively true of the original strains of both species and of a number of strains obtained by dissociation. Strain C<sub>2</sub> of *B. mycoides* has a strictly equatorial germination in various media, and seldom exhibits lateral deviation even in a nitrogen-free medium. The author knows of no other single character, morphological or physiological, commonly used in the classification of the genus *Bacillus* which shows a greater degree of constancy than the mode of germination. It is an easily determinable morphological character which has been correlated with antigenic make-up and physiological behavior (107, 108, 101). However, by present standards of taxonomy, it will serve only as a group character. Those who discount its use do not seem able to show any valid cause for their aversion other than to state that the mode of germination is variable; that it could not be

dependable since it depends on slight differences in the character of the coats. In reality, the remarkable constancy of the mode of germination in a given strain could not be accidental; and it will probably be related to the manner in which the coats are laid out as suggested by the work of Schaudinn (153), and to an intimate relation between the coats of the spore and its protoplasm. The form and size of the emerging germ cell, used by Gottheil (67) as a related criterion, should be reinvestigated.

#### BIOLOGICAL NATURE

The biological nature of the endospore has not yet been established. The following quotations from authoritative writings which appeared during the past fifteen years illustrate present beliefs. Cook (25) states: "There is no doubt that bacterial spores are not necessarily formed because conditions in the environment have become unfavorable for further growth. It is extremely likely that they represent a stage in the life cycle of certain bacteria. . . . The function of spores is by no means clear, but they possibly represent a survival from a parent form. As these bodies are heat and drought resistant, they are undoubtedly in many instances a factor in the survival and distribution of the race. . . . They are specifically produced to tide over unfavorable periods." According to Henrici (74): "Only one spore is formed by each cell, and upon germination each spore gives rise to but one cell. They are, therefore, not reproductive in function but are essentially *resting forms*." A similar statement is made by Zinsser and Bayne-Jones (174); these authors, however, add: ". . . we are convinced from our observations of the process that the formation of the endospore has a significance in the bacterial life cycle greater than its obviously advantageous production of a temporarily resistant form." Lewis (115) places endospores among reproductive structures but does not otherwise discuss their biological nature.

It is generally believed that endospores are formed asexually; however, one finds in the literature several reports in which autogamic, sexual processes are said to take place during its formation, at least in certain species. Schaudinn (153, 154) interpreted the processes he described in *B. bütschlii* and *B. sporonema* as evidence of autogamy. Guilliermond (70), who studied several of the aerobic sporeformers, wrote: "Sporulation does not seem to be preceded by phenomena of autogamy similar to those described by Schaudinn in *B. bütschlii* and *B. sporonema*. It is, therefore, probable that the phenomena of autogamy are far from being the general rule in bacteria." (Translated by the reviewer.) Dobell (46) severely criticized Schaudinn's interpretation. In *B. flexilis*, a disporic organism in which the spores are formed as in *B. bütschlii*, Dobell saw evidence of incomplete cell division rather than of autogamy.

Badian (3, 4) described sporulation in *B. subtilis*, *B. mycoides*, and *B. megatherium* in the following manner: The vegetative cell usually contains a single chromatin rod. Once vegetative development is completed, the cell enters the phase of autogamy. Autogamy is preceded by longitudinal division of the chromosome into two secondary chromosomes which are oriented parallel to the



long axis of the cell and which fuse at their proximal ends forming a single thread. This thread becomes shorter and thicker, finally appearing as a typical chromosome. This is the beginning of the diplophase. The bivalent chromosome then shifts into an oblique, or transverse, position and initiates a new series of cell divisions. Formation of the endospore is preceded by a double division of the chromosome. Three of these chromosomes disintegrate, and only one forms a part of the spore. Disintegration of the three chromosomes indicates that the division of chromosome preceding spore formation is a reduction division. The endospore, therefore, contains a single, univalent chromosome and is the beginning of the haplophase.

Allen *et al.* (1) observed three methods of spore formation in an aerobic organism isolated from grass. In one of these methods, the authors see evidence of nuclear reorganization. "The main changes involved are explained on the assumption that the normal vegetative cell is haploid and contains one chromosome. Prior to one type of spore formation a diploid cell is thought to be formed by division of the chromosome, accompanied by a failure in cell division. Meiosis then occurs resulting in the formation of a well-marked, deeply staining tetrad, followed by separation of the chromosomes and possibly a second mitosis. One of the resultant chromosomes is involved in the formation of the spore and the remainder appear to be extruded. The spore is thus haploid in constitution. On the other hand some spores appear to be formed from similar cells without previous meiosis, and it is thought that in these spores, which would be diploid, the meiosis which was repressed at spore formation occurs either when the liberated spores are sown on to a fresh medium, or when they are left for some time in an old medium."

Klieneberger-Nobel (84), using the Pickarski-Robinow technique of hydrolysis with HCl and staining with Giemsa's solution, studied the cytology of sporulation in *Clostridium welchii*, *C. septicum*, *C. oedematiens*, *Bacillus mycoides*, and *Sphaerotilus natans*. She summarizes her interpretations as follows: "The dumbbell bodies which are dispersed in the cells of young growth become aligned in the long axis of the cell where they eventually fuse into an axial nuclear cylinder. These cells divide up into fusion cells of approximately the same length. The development of the 'chromosome' stage into the fusion cell is the first step in the process of sporulation. During its further development the fusion cell or spore mother cell divides twice with the result that it is segregated into four structures which often assume dumbbell shape. Therefore the chromatin cylinder of the individual spore mother cell seems to be equivalent to four nuclear elements one of which functions as the spore 'chromosome', ('nucleus?'), whereas the remaining three disintegrate. The ripe spore representing, as it does, the smallest cell unit contains one nuclear structure only."

"Therefore the two main features in spore formation of bacteria appear to be 1, a fusion of the dumbbell bodies into an axial chromatin rod ('autogamy?'); 2, a reduction partition which is reminiscent of, though not corresponding to, the more complicated phenomenon of meiosis in the higher organisms." It is easy to see that these interpretations are identical with those of Badian (3, 4). Other papers on the nature of the endospore are 118, 149, and 150.

*Discussion.* A critical study of the literature reviewed in this section leads to the same statement with which the section was started, *i.e.*, "The biological nature of the endospore has not yet been established." In the literature, we find two types of assumed automictic processes: the first involves the fusion of sister cells; the second involves the fusion of nuclei, or "chromosomes," present in the same cell. The first, *i.e.*, fusion of sister cells, was observed by only one investigator in organisms not available for study by others. However, the observations of Schaudinn appear to be of such a quality that, in spite of criticism (46) one can not discount the process as a possibility in some unusual bacteria. It must be emphasized, however, that this process has not been observed in common bacteria. Since it involves easily recognizable morphological criteria, namely, division of a cell followed by disappearance of the septum, its occurrence could not have escaped the notice of other investigators (5, 97) who made "continuous" observations on numerous sporulating cells. The second, *i.e.*, fusion of the nuclei in the same cell, is more difficult to evaluate, since it is reconstructed from the observation of minute structures in cells subjected to considerable manipulation. The deductions made are based on three assumptions the correctness of which has been taken for granted by the respective authors. The first assumption is that wet fixation of the cell with osmic acid and subsequent treatment with hot, normal hydrochloric acid do not, in any way, alter its structure; the second is that Giemsa's stain is specific for chromatin; the third is that a morphological picture observed, or more particularly photographed, with the light microscope can be mechanically interpreted without consideration of optical relationships.

The reviewer has, so far, refrained from evaluating the stain of Giemsa in the study of the internal structure of bacteria. Its use following wet fixation with osmic acid was highly recommended and extensively used by Dobell (47). Badian (3, 4) observed internal differentiation in cells, stained by Giemsa's solution and decolorized by eosin. Piekarski (139) used the stain followed by decolorization with eosin or preceded by treatment with hydrochloric acid as in the Feulgen technique; both methods gave similar pictures. Piekarski believed that action of the acid consisted in loosening the structure of the membrane, thus increasing its permeability to the dye. Robinow (147, 148) combined the methods used by Dobell and Piekarski; the wet cells were fixed with osmic acid, treated with normal hydrochloric acid as in the Feulgen procedure, and stained with Giemsa's solution. He attributed to the acid reduction in the stainability of the cytoplasm. All of these investigators were able to observe intracellular bodies which stain as known nuclei do, although the morphology, number, and distribution of these bodies may have been widely different. The illustrations published by Robinow show many of these bodies with a dumbbell-like form, usually interpreted as evidence of division.

The possibility that the observed bodies are nuclei can not be doubted, since Giemsa's solution has been used in histology as a nuclear stain for many years. On the other hand, the problem with bacteria is somewhat different. Here the problem does not involve only demonstration of bodies which stain as nuclei, but also proof that such bodies were not formed by fixation or hydrolysis with

acid from nuclear material not differentiated in the living cell as nuclear bodies; it also involves demonstrating that the bacterial cell contains no other bodies which stain like nuclei. In this connection it may be recalled that the fat-containing inclusions formed by *B. cereus* in the prespore stage and the cytoplasmic membrane, in which these inclusions are formed, give a positive Feulgen reaction (93, 95). The cytoplasmic membrane is also colored by Giemsa's solution, with or without hydrolysis, in a shade similar to that of the nucleus. Since Knaysi and Baker (98) showed that, in nonfilamentous growth of *B. mycoides*, all the nuclei of a sporangium are enclosed in the forespore, the extrasporal bodies considered by Badian (3, 4) and by Klieneberger-Nobel (84) as supplementary nuclei must be prespore inclusions. Furthermore, dumbbell-like appearance should not be considered a proof, but only an evidence, of possible division, for two bodies accidentally lying side by side would appear as one with a dumbbell-like form; only by the use of probabilities and by correlation with cell development can this phenomenon be given significance. We are also unable to agree with Piekarski (139) that hydrolysis produces no artifacts; for even if we accept pre-existence of the stained bodies in the living cell, we still have to consider artificial positions and arrangement which, as was pointed out in the preceding section, are observed in cells subjected to hydrochloric acid. In an unpublished work (94), it was possible for us to treat a given cell, under the microscope, in such a way that the positions of its inclusions changed considerably.

It is clear from this discussion that use of Giemsa's solution, with or without hydrolysis with acid, did not solve the problem of internal structure in the vegetative cell or in the endospore, and that indiscriminate use of this technique has already led, and will continue to lead, to erroneous interpretation of internal structure and of nuclear behavior. Theoretically, staining with Giemsa's solution is of no more significance than staining, for instance, with hematoxylin. On the other hand, the acid treatment, transplanted into the technique from the Feulgen procedure by Piekarski (139), has the advantage of removing the most troublesome substance, ribonucleic acid (89). This, added to the excellent optical qualities of the stain, is of considerable advantage in making and recording observations. However, its significance depends on the ability of the observer to show, by other means, that non-nuclear, Giemsa-stainable bodies are not present in the object examined. This, the users of the method have failed to do. On the other hand, the work of Knaysi (94) and that of Knaysi and Baker (98) have shown that young, actively growing cells of *B. cereus* and *B. mycoides* contain no intracytoplasmic bodies other than nuclear ones; consequently, Giemsa-stainable bodies in young cells of these organisms probably consist of chromatin. In making this statement, we are assuming that these bodies are not formed by coagulation of diffuse nuclear material; but even with young, actively growing cells, it is risky to draw conclusions with respect to nuclear behavior, because of artificial positions or arrangements caused by the technique. However, at the end of the period of growth, cells of these organisms contain Giemsa-stainable inclusions consisting of protein combined with lipid

material and possibly other molecules (93, 94). These bodies originate in the cytoplasmic membrane and have no nuclear significance; their staining properties are not altered by treatment with acid. Under these conditions, the technique is of a very limited value. These considerations, together with the observation (98) that all the nuclei of a sporangium are enclosed in the forespore, show that the scheme of nuclear behavior before and during sporulation, as visualized by Badian and by Klieneberger-Nobel, is based on uncritical interpretation of staining with Giemsa's solution. It must be emphasized, however, that the possibility of autogamic processes within the spore is still to be considered.

#### CHEMICAL COMPOSITION AND ANTIGENIC STRUCTURE

*Chemical composition.* Little is known about the chemical composition of the endospore other than that it contains a number of enzymes (52, 53, 152, 24, 163, 167), more bound water and less free water than the vegetative cell (64, 75), mineral elements such as Ca, K, Cu, and Mn (28), and ribonucleic acid diffuse throughout the cytoplasm and utilizable as a source of nitrogen and phosphorus but, in the case of *B. mycoides*, not of energy (92, 98). It may be added that the cytoplasm of the spore is rich in lipid material, also diffuse. Since the spore contains nuclei, it also contains the nucleoproteins characteristic of the nucleus.

*Antigens.* Defalle (37), using several members of *Bacillus* (*B. mycoides*, *B. mesentericus vulgatus*, *B. subtilis*, *B. alvei*, and *B. anthracis* attenuated with phenol), observed that the injection of spores of these bacteria into the animal body was followed by the formation of antibodies (agglutinins and sensitizing antibodies) in the serum, whereas the injection of mold spores did not confer any special property on the serum. He further showed that the formation of antibodies was not the result of germination but was induced by the spores themselves; that the antibodies induced by spores of one species were more active against the spores of that species, but acted also on the spores of other species; that the antibodies produced against the spores differed from those produced against the vegetative cells in that injection of spores heated to 115 C induced the formation of both agglutinins and sensitizing antibodies, whereas the injection of vegetative cells heated to the same temperature induced the formation of agglutinins only.

Mellon and Anderson (119), working with spores of *B. subtilis* treated with antiformin, confirmed the existence of spore antigens distinct from those of the vegetative cell. On the other hand, Krauskopf and McCoy (105) found a close serological relationship between spores, treated with KOH to dissolve vegetative remnants, and vegetative cells. "Absorption experiments revealed the presence of an H (flagellar) factor in spores, demonstrable both *in vivo* and *in vitro* reactions."

Lamanna (107, 108) also confirmed the existence, in many strains of *Bacillus*, of a separate spore antigen. "The antibody obtained by injection of spores was specific. It did not react with vegetative cells of the same strain. The vegetative cells of the homologous organisms do not absorb out the spore antibody. It will be noted that in some instances the spore antiserum clumps vegetative

cells along with the spores. . . . Upon absorption with vegetative cells the serum loses the ability to clump them and the titer for the spores remains unaffected." Lamanna correlated antigenicity of the spore with other morphological and physiological characteristics and used it in the taxonomy of the genus *Bacillus*. Strains with small cells were divided into four, well defined groups. In strains with large cells, it was possible to separate *B. megatherium* from *B. cereus*, but *B. mycoides* proved to be heterogeneous. In a later paper, Lamanna (110) prepared precipitinogens from the spores of *B. subtilis* and *B. vulgaris* (i.e., the strain of Ford and Lawrence and the Marburg strain of *B. subtilis* respectively). He found that: "The antigen of each species does not cross precipitate with spore antiserum of the heterologous species."

Howie and Cruickshank (77) worked with *Clostridium sporogenes*, *B. cereus*, *B. mesentericus*, and two other members of *Bacillus* isolated from soil. "Antisera against *Cl. sporogenes*, *B. cereus*, and the organism 'Soil 1' were prepared by injecting suspensions largely composed of spores but in which small numbers of bacilli were also present. These antisera contained agglutinins against both spores and bacilli but by absorption with heavy suspensions of the appropriate antigen it was possible to remove one agglutinin without appreciably lowering the titer of the other. . . . The organism 'Soil 2' when grown for 10 days on sodium asparaginate agar produced growths containing spores and so few bacilli that the latter were unable to stimulate antibody formation. By this means a spore antiserum was obtained which agglutinated spores but not bacilli. . . . Antisera were prepared against autoclaved and unautoclaved suspensions of *B. mesentericus* containing few bacillary forms. Autoclaving destroyed the antigenic activity of the bacilli but did not significantly alter the antigenic properties of the spores." This paper contains a critical evaluation of previous work on the subject. A recent paper by Bekker (8) reports similar conclusions.

On the basis of the work reported above, it seems fairly certain that the endospore has at least one antigen not present in the vegetative cell. Since agglutinogens are surface antigens, this is supported by the difference observed in electrophoretic mobility between the spore and the vegetative cell. Furthermore, it seems that the characteristic spore antigen is not the same in all spore forming bacteria, and that organisms of the same serological group have also several morphological and physiological characteristics in common. In view of the sad state of the taxonomy of the sporeformers, it is hoped that further study of spore antigens will prove helpful in developing a sound knowledge of this interesting and important group of bacteria.

#### RELATION OF THE ENVIRONMENT TO THE FORMATION AND GERMINATION OF THE ENDOSPORE

##### *Formation*

Although the endospore represents a stage in the normal development of certain bacteria, its formation is considerably affected by the environment. Under certain conditions, it is possible to grow a strain of bacteria potentially

able to form spores, in the vegetative stage indefinitely. "Permanently" sporeless strains may also be developed by dissociation (130, 79, 111, 7, 151, 136-138, 56, 57, 85, 86).

The environmental factors which affect sporulation have been extensively investigated during the past sixty years, but, until recently, the state of knowledge in that respect was fragmentary and short-sighted. The reasons will be given below. The environmental factors which have been investigated are the following: the temperature of incubation; the composition of the medium with respect to type and initial concentration of gross nutrients, or the accumulation of byproducts, molecular oxygen, salts (particularly cations, including hydrogen ion), the presence of nitrates, and the accumulation of byproducts of metabolism; desiccation of the medium; finally, the stage of development of the culture with reference to the "growth curve."

1. *The temperature of incubation.* It was reported by Cohn (23) that *B. subtilis* grows actively at 47 to 50 C, forming both a pellicle and spores in the normal way. Between 50 and 55 C, however, growth ceases; neither pellicle nor spores are formed, and the vegetative cells already present are destroyed. Koch (104) studied the effect of temperature on *B. anthracis*. At 35 C the organism grows fastest and spores are formed in less than 20 hours; at 30 C, the spores appear in about 30 hours; and at 18 to 20 C in 2½ days. Spores are seldom formed below 18 C, and growth ceases below 12 C.

Brefeld (10) found that *B. subtilis* divides at 24 R in a half hour, at 20 in three-quarters of an hour, at 15 in 1½ hours, and at 10 in 4 to 5 hours; below 5 R, growth was extremely slow. Spore formation takes place at 24 R in about 12 hours, at 18 in one day, at 15 in two days, and at 10 in several days. The cycle of development (probably of the culture) is completed at 24 R in 24 to 30 hours, at 20 in 2 days, and at 15 in 4 to 5 days.

Schreiber (155) concluded, from his studies of *B. anthracis*, *B. subtilis*, and *B. tumescens*, that the effect of temperature on sporulation is slight and is due to extension of vegetative growth. At the optimum temperatures (34 C for *B. anthracis*, 30 C for *B. subtilis* and *B. tumescens*), spore formation takes place early. In *B. subtilis*, vegetative growth ceases at 8 C and sporulation at 10 C; in *B. tumescens*, growth ceases at 10 C and sporulation at 11 C; *B. anthracis* grows slightly at 12 C but spores are not formed below 14 C. Of considerable interest is the observation that when a 14-hour-old culture of *B. anthracis* is removed from 37 to 18 C, involution forms are produced and sporulation is deficient; when the change in temperature is not so great, say from 30 to 20 C, no injury results other than retardation of the rate of growth.

Migula (123, 125) determined the periods it takes *B. subtilis* to sporulate at various temperatures. At 14 C, the period was 72 hours; at 18 C, 54 hours; at 20 C, 48 hours; at 25 C, 40 hours; at 30 C, 33 hours; at 35 C, 26 hours; at 38 C, 22 hours; at 40 C, 38 hours. Migula concluded that the temperature of fastest growth corresponds to the minimum period for sporulation, since, at this temperature, the medium becomes most quickly unsuitable for vegetative growth. On the other hand, spores are not formed at all temperatures which allow vege-

tative growth; for instance, *B. subtilis* forms well-developed colonies within a week at 4 to 8 C, but spores are never formed in this interval. A similar observation was made on *B. anthracis*.

Blau (9), a student of Arthur Meyer, determined the maximum temperatures for spore germination, for multiplication, and for spore formation of 24 mesophilic and 4 thermophilic species of sporeformers. In ten species, including *B. mycoides*, *B. ellenbachensis*, and *Sarcina ureae*, the three maxima in the same species were the same; in *B. alvei*, the maximum for spore formation (45 to 50 C) was higher than that for spore germination (40 to 45 C); in the remaining 17 species, the maximum for spore formation was below that for germination.

More intensive and, apparently, exact studies of the effect of temperature on spore germination, growth, and spore formation were made by Holzmüller (76). The organisms investigated were 5 strains of *B. mycoides* and 4 other species of sporeformers. The cardinal points of each of the three processes varied with the strain. For any given strain, germination, growth, and spore formation had the same optimum, but germination took place within a narrower interval than growth, and spore formation in a narrower interval than germination. Holzmüller expressed disagreement with Blau (9) who had reported that the maximum temperatures for all three processes in *B. mycoides* were the same (30 to 35 C). In reality, Blau reported his observations for 5-degree intervals and his conclusions are justified only within the limits of these intervals. Thus, whenever the three maxima fell within the same interval they were reported the same. Indeed, Blau's conclusions went beyond the accuracy of his results. If this is taken into consideration, the presumed disagreement vanishes. The surprisingly low maxima reported by Blau for *B. mycoides* must be ascribed to the strain he investigated, since Holzmüller reported as much as 5 C difference between maximum temperatures of sporulation in different strains of the same organism. *B. mycoides* is undoubtedly a group of species having in common chiefly the well known type of colony.

Of considerable interest are the observations of Christian (21) on a *Bacillus* which causes "coconut" or "carbolic" taint in heated milk. The cardinal points for vegetative growth and spore germination are as follows: the minimum is about 5 C, the optimum between 30 and 37 C, and the maximum is about 56 C. For spore formation, however, the optimum is 22 C; at 30 C occasional spores are formed; at 37 C no spores are formed. Of possible importance is the observation that at 37 C the organism shows "evidence of an effort at spore formation. The cell contents concentrate in a terminal position as in the case of the formation of true spores, but no spore wall is formed. Later, these terminal bodies or 'abortive spores' are liberated and persist in the cultures in the free state for long periods. It has not yet been demonstrated whether they are capable of developing further under suitable conditions or of giving rise to new generations of the organism." One wonders whether or not similar bodies recently observed in certain thermophilic bacteria (Buck, 13, and personal communication) are abortive spores which may attain full development below the optimum temperature for vegetative growth.

2. *Composition of the medium.* a. *Initial concentration of gross nutrients and accumulation of byproducts.* Brefeld (10) stated that when the nutrient solution is exhausted *B. subtilis* proceeds to form spores. Lehmann (112) and his student Osborne (131) found that, when *B. anthracis* is grown in aerated broth diluted to various extents, the numbers of vegetative cells as well as of spores are proportional to the initial concentration of the broth. On nutrient agar previously exhausted by the growth of successive crops of the same organism, the numbers of both vegetative cells and spores are slight. Buchner (12) observed that *B. anthracis* may be kept indefinitely in the vegetative stage by periodic transfer, at a suitable interval, into a fresh medium, and that when vegetative cells are transferred into distilled water and incubated under conditions suitable for sporulation, spores are formed sooner than in the culture from which the transfer was made. The same was true of *B. subtilis*. Inability of the anthrax organism to form spores in the animal body is attributed not only to the absence of molecular oxygen but also to growth under conditions where nutrients are never exhausted. Turro (165), also working with *B. anthracis*, was able to induce germination of the spores and fresh vegetative growth by neutralizing the acids of fermentation with sodium hydroxide. He concluded that sporulation is caused by the accumulation of certain byproducts of metabolism.

Schreiber (155) repeated and extended Buchner's experiments, using *B. anthracis*, *B. subtilis*, and *B. tumescens*. He drew the following conclusions: 1, spores are never formed under conditions of continuous, active growth; 2, insufficient food and unfavorable environmental conditions hinder or prevent spore formation; 3, sudden hindrance of growth following good nutrition causes immediate and complete sporulation; 4, substances which hinder growth and, consequently, favor sporulation are sodium carbonate, magnesium sulfate, sodium chlorate, and distilled water. Migula (123, 125) observed that addition of dry peptone and meat extract to a broth culture of *B. anthracis* shortly before sporulation does not stop the process. If, on the other hand, the medium is simultaneously diluted with distilled water, further vegetative growth is induced and sporulation is delayed. He concluded that sporulation is not due to exhaustion of the medium. Stephanidis (160), a student of Lehmann, found that spore formation in *B. anthracis* took place sooner on poor than on rich media. On the basis of the average number of spores per chain of cells grown on nutrient agar in which the concentration of meat extract varied from 5 to 0.02 per cent he concluded that both the relative and absolute numbers of spores increased with the concentration. Holzmüller (76) concluded, from a study of 5 strains of *B. mycoides* and of 4 other species of *Bacillus* that sporulation requires good growth followed by deficiency of food. It is prevented by continuously good nutritional conditions and hindered in a poor medium and by conditions unfavorable for growth. Möhrke (127), studying two or more strains of *Clostridium tetani*, *C. botulinum*, *C. oedematiens*, and *C. putrificum*, found that media rich in protein, such as serum agar or serum agar + alkali albuminate, promoted sporulation of these organisms.

Henrici (73) inoculated 4 sets of agar slants with spores of *B. cohaerens*; two of



the slants contained nutrient agar of normal composition, the other two contained nutrient agar with one-quarter as much peptone and meat extract. One set of each kind was inoculated with a very heavy suspension of spores, and the other with the same suspension diluted 1/50. "Spore formation proceeded more rapidly in the media of lower nutrient value than in the full strength media, regardless of the size of seeding, . . . and it proceeded more rapidly in the heavily seeded cultures than in the lightly seeded ones, though the difference here was not so pronounced as was the effect produced by varying concentration of nutrients. In the heavily seeded dilute medium, spores never entirely disappeared, new spores starting to form before all of those introduced had germinated. It would appear, therefore, that the rate of spore formation is determined not by the concentration of cells alone, but rather by the density of the population in relation to the concentration of foodstuff in the medium."

Williams (169-171) was unable to get satisfactory spore yields with *B. subtilis* in several synthetic media. However, when the organism was grown in shallow layers of peptone solutions (1 to 5 per cent), the percentage of spores varied inversely with the concentration of peptone, although the total number of spores was higher in the more concentrated solutions. Brunstetter and Magoon (11) also reported that when *B. mycoides* is grown in aerated peptone solutions, the percentage of spores at the end of one day decreased as the concentration of peptone increased; the opposite was true of *B. fusiformis*. From these observations it was concluded that "both the amount of available food and the amount of metabolic products in the environment are of great importance in determining the extent of sporulation by *B. mycoides*, . . ." whereas with *B. fusiformis* "The accumulation of metabolic products seemed to be of much more importance than the food supply." See also Tarr (162). Bayne-Jones and Petrilli (5) stated that "it was always necessary to provide an initially adequate medium for the growth of vegetative cells, and we noted, in general, that an unfavorable environment inhibited spore-formation."

Kaplan and Williams (81) who studied *Clostridium sporogenes* concluded that "An increase in the concentration of peptone beyond a necessary minimum does not greatly affect the rate of formation of spores . . . ; variations in the meat extract content of the medium have even less influence, spores being formed in almost the complete absence of this ingredient. . . . The presence of 1 per cent fermentable sugar in dilute nutrient agar results in almost complete inhibition of spore formation. The presence of 1 per cent lactose stimulates the rate of sporulation. . . . In an originally alkaline medium, the presence of 1 per cent fermentable sugar inhibits sporulation only in media which contain small concentrations of available nitrogen." The harmful effect of fermentable sugars is readily explainable by the formation of acids. The unfermented lactose exerts a beneficial effect probably by its reducing action.

Knaysi (91) showed that strain C<sub>2</sub> of *B. mycoides* can grow and form endospores on 1 per cent bacto agar, prepared with distilled water, to which no nutrients were added. He also showed that in dilute media the number of endospores is proportional to the concentration of nutrients. Beyond a certain

concentration, the number of endospores increases with the concentration but is not proportional to it. Barring toxicity, which has been often reported for concentrated peptone solutions, the reason for this apparently harmful effect of concentration must be sought in the fact that, for a given slant surface, the more concentrated slant gives a thicker layer of cells; below the surface of this layer, the environment varies from semiaerobic to anaerobic and, therefore, is unsuitable for sporulation. It can be easily shown that the numbers of endospores and of vegetative cells in slant cultures of the same initial composition are, at least within a certain period, functions of the ratio between the free surface area and the volume of the slant."

*b. Molecular oxygen.* Referring to *B. subtilis*, Cohn (23) stated that the bacilli carry on fermentation in an air-free environment whereas intensive growth and spore formation are bound to an unhindered access of air. Koch (104) showed that spore formation by *B. anthracis* requires air, moisture, and a temperature exceeding 15 C. Brefeld (10) stated that spore formation by *B. subtilis* in a nutrient solution is general and regular only when the organism grows as a pellicle in contact with the air. When growth is entirely within the liquid, spore formation is delayed. Contamination with other bacteria prevents the formation of a pellicle and, consequently, prevents or hinders the formation of spores. Buchner (12) was also aware of the importance of molecular oxygen for *B. anthracis* and *B. subtilis* not because of any specific action in the formation of the spore but because of its beneficial effect on vegetative growth.

Schreiber (155) found that, since *B. anthracis* is not motile and grows at the bottom of a nutrient solution, the formation of spores by this organism is hindered, or prevented, in a tube where height of the solution exceeds 15 cm. Experiments with *B. subtilis* and *B. tumescens* in which the tubes were stoppered with cork and paraffin showed that, for normal development, *B. subtilis* required at least 3 cm of air above the liquid column, and *B. tumescens* 5 cm. Schreiber attributed to molecular oxygen a specific action in spore formation. He reasoned as follows: Bound oxygen is utilized, to a limited extent, for vegetative growth; but, even in the richest media, spores are not formed without free oxygen and, following exhaustion of the media, the cells perish. Limitations of this type of reasoning will be pointed out later.

Matzuchita (117) studied the effect of oxygen concentration on the growth and spore formation of a number of aerobic and anaerobic bacteria on agar, gelatin and, sometimes potato. He incubated his cultures under a bell jar where the air pressure and the oxygen content could be measured, and where measured volumes of hydrogen could be introduced at definite pressures. Some of his results, such as massive growth of *B. subtilis* where the oxygen content was extremely small, suggest, according to Wund (173) that either his apparatus was not sufficiently air-tight or that the hydrogen he used was not sufficiently pure.

Migula (125) stated that the oxygen requirement of the same organism varies with the conditions. He said that Weil reported sporulation by the anthrax organism under an atmosphere of hydrogen when the organism was grown on potato, wheat infusion, quince, marshmallow, or coagulated sheep serum to which

bouillon, containing 25 per cent of glucose, was added, but not in other media. He further stated that Klett reported spore formation in ordinary media under an atmosphere of nitrogen, and that Jacobitz attributed these results to lack of removal of oxygen from the medium. Migula himself claimed that sporulation of anaerobes, once begun, is hastened, or at least not hindered, by the access of a certain amount of oxygen, and that action of the oxygen here is similar to that of the products of metabolism, rendering the medium unsuitable for vegetative growth.

Wund (173), a student of A. Meyer, determined what he called the cardinal points of oxygen concentration for germination, growth, and spore formation by a number of sporeforming bacteria of the genus *Bacillus*. He concluded that the maximum concentration for spore formation is usually below, and never above, that for spore germination; that the minimum for spore formation is usually above, and never below, that for germination. A similar relation holds for spore formation and growth. He further concluded that the minima for growth and germination are the same, whereas the maximum for growth may be equal to that for germination or be below it. Only in the case of *Bacillus alvei* is the maximum for growth above that for germination. Wund's paper contains many tables which should be consulted for exact values of the cardinal points for a given organism. Holzmüller (76) concluded that oxygen is not necessary for the germination of spores. It is necessary for the formation of spores by aerobic, but not always by facultative, bacteria.

The effect of low oxygen pressures on the growth and sporulation of a number of aerobic and anaerobic bacteria was studied by Leifson (113). The anaerobes showed "a considerable difference in their ability to sporulate under increased oxygen tensions. The sporulation of *Cl. tetani* and *Cl. novyi* was inhibited at 1 cm of oxygen. All the others except *Cl. sporogenes* and *Cl. chauwei* sporulated less at 1 cm of oxygen than at 0 cm. . . . The sporulation of all the organisms was inhibited by 2 cm of oxygen. In the case of the aerobes no sporulation was observed at 2 cm of oxygen although fair growth was obtained. *B. circulans* seems to be the most 'anaerobic' of the aerobes used—growing as it did in less than 1 cm of oxygen."

Bayne-Jones and Petrilli (5) noted with *B. megatherium* "that an adequate supply of oxygen in proportion to the amount of growth was more important than the exhaustion of the medium" for spore formation.

Knaysi (91) found, with *B. mycoides*, that when the rate of aeration of liquid cultures is slow, the formation of endospores is slow and their numbers remain relatively small. "As the rate of aeration increases, endospores are formed sooner and in greater numbers. . . . When glucose is present in the medium, a slow rate of aeration results in an extension of the period of purely vegetative growth. Sporulation may be hastened either by a reduction of glucose or by an increase in the rate of aeration, and, unless the concentration of glucose is low, it may be difficult to aerate with sufficient vigor to promote sporulation within a reasonable time." In glucose broth cultures sealed with vaseline-paraffin, relatively few endospores were found in cultures, several months old, of *B. subtilis*

and *B. atterimus*. In test tubes 150 by 15 mm closed with a rubber stopper, a 5-ml slant culture of *B. mycoides* on glucose agar contains practically no spores; the relatively few spores formed owing to temporary shortage of food germinate as more nutrients diffuse to the surface, and only a few shells can be observed in such cultures. In view of the relatively considerable quantities of oxygen consumed during vegetative growth, the minimum oxygen requirements reported for endospore formation must be taken with caution, unless it is shown that the recorded initial pressure remained the same at the time of sporulation. Knaysi believes that the absolute necessity of molecular oxygen for sporulation of *Bacillus* spp. has not yet been conclusively proved, although its necessity for the quick and efficient formation of large numbers of spores is "one of the incontrovertible facts of bacteriology."

c. *Salts and ions*. Behring (6) stated that sporulation, in *B. anthracis*, was stimulated by definite amounts of lime water or  $\text{CaCl}_2$  and inhibited by larger amounts. Schreiber (155) investigated the influence of a number of salts on sporulation in *B. anthracis*, *B. subtilis*, and *B. tumescens*. The vegetative cells were harvested from nutrient agar cultures of a definite age and suspended in distilled water or in solutions of the various salts tested. In 2 per cent sodium chloride spores appeared in 10 to 12 hours; in 2 per cent sodium carbonate or 1 per cent magnesium sulfate, in 8 to 10 hours; in 2 per cent potassium nitrate or 3 per cent glycerol in 18 to 20 hours. This is to be compared to 14 hours in distilled water.

Fitzgerald (62), working with *B. capsulatus*, observed no stimulating effect on sporulation by 0.5 to 5.0 per cent sodium chloride. Cook (24) tested the effect of  $\text{NaCl}$ , in concentrations ranging from 0.5 to 5 molar, on growth and spore formation in *B. subtilis*, but could not definitely state that any one concentration was favorable for spore formation. Fabian and Bryan (61) studied the effect of cations on spore formation in *B. subtilis*, *B. cereus*, *B. mesentericus*, and *B. megatherium*. Cations of univalent chloride salts,  $\text{NaCl}$ ,  $\text{LiCl}$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{KCl}$ , and sodium lactate exerted a distinct stimulation in a liquid medium. Cations of the bivalent chloride salts,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{BaCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{PbCl}_2$ ,  $\text{NiCl}_2$ , the trivalent chloridesalts  $\text{AlCl}_3$ ,  $\text{CeCl}_3$ ,  $\text{FeCl}_3$ , and the tetravalent chloride salt  $\text{SnCl}_4$  had no stimulating effect. Knaysi (91), working with *B. mycoides*, found that  $\text{MgSO}_4$  increases the vegetative population. It has a beneficial effect on sporulation when the oxygen supply is low or limited.

d. *The hydrogen ion*. Behring (7) stated that hydrochloric acid, or rosolic acid, added in definite amounts to the medium, inhibit sporulation and may lead to asporogenous strains. Schreiber (155) studied the effect of alkaline reaction by adding various amounts of sodium carbonate to the medium. The optimum for *B. anthracis* was 0.5 to 1.0 per cent and the maximum 3.0 per cent; for *B. subtilis* the optimum was 2.0 per cent and the maximum 5.0 per cent. The acid reaction was produced by the addition of tartaric acid. *B. anthracis* tolerated 0.3 per cent; *B. subtilis* and *B. tumescens* 1.0 per cent. At the acid reactions, the intensity of sporulation was found very slight.

Fitzgerald (62) concluded from a study of *Bacillus aerogenes capsulatus* that

"The presence of acid, either as a primary constituent of the medium, or as the resultant of bacterial action upon the sugar contained in the same, appears to have an inhibitory effect upon sporulation, but this effect is not absolute, since spores were occasionally met with in the media containing HCl, and in one instance were also formed in the presence of formed acid due to action upon raffinose. . . . Alkaline media are more conducive to sporulation than either acid or neutral media, and appear to be necessary for its occurrence in any high degree."

Simonds (157, 158) stated that, in *Bacillus welchii*, "spore formation is inconstant and occurs only in alkaline media, never in pure cultures containing a fermentable sugar or a free acid." On the other hand, "in the presence of the mixed fecal flora, the bacillus welchii may sporulate even in the presence of free acid or fermentable carbohydrate." The limit of acidity which allows sporulation under the latter condition is equivalent to 3 ml of N/10 NaOH per 100 ml of suspension, using phenolphthalein as an indicator.

Itano and Neill (80) found that *B. subtilis* can complete its life cycle between pH 5 and pH 10; its pH limits for growth were 4.2 and 9.4. At pH 10, spore germination is followed by slight growth and return to the spore stage. "The slight growth and multiplication of vegetative cells in broth of pH 10 suggest that the formation of endospores in this medium must be caused largely by the unfavorable reaction of the medium rather than by the accumulation of end products."

The optimum pH for sporulation of *B. sporogenes* is, according to de Smidt (42), 7.7-7.9. Möhrke (127) found that the optimum pH for sporulation, in two or more strains of *Clostridium tetani*, *C. botulinum*, *C. oedematiens*, and *C. putrificum*, is about 7.1. A slightly acid reaction is still suitable, but a slightly alkaline reaction is injurious to sporulation.

Torrey, Kahn, and Salinger (164) showed that in a sugar-free, well buffered fluid medium, the favorable zone for spore formation by *Bacillus welchii* "ranges from pH 6.8 to the alkaline limit for its growth with an optimal reaction between 7.4 and 8.0. Spore formation is entirely checked during the first week in reactions more acid than pH 6.6. . . . The high *B. welchii* spore counts for fecal specimens from cases with gastric anacidity (including pernicious anemia) may be explained by the fact that the reaction of the small intestine in such cases is favorable for both spore formation and active growth of this organism."

Cook (24) stated that, in media buffered at definite pH ranging from 3 to 9, "growth and spore formation" of *B. subtilis* "were only noted in the media at pHs 6 and 7." Fabian and Bryan (61), working with *B. subtilis*, *B. cereus*, *B. mesentericus*, and *B. megatherium* found that "The pH of the medium studied did not materially affect the formation of spores within a favorable growing range pH 5.0 to 7.5. However, an acid reaction was slightly more favorable for their production." Kaplan and Williams (81) observed growth of *C. sporogenes* at pH 5.7 but not at 5.3. Some sporulation takes place whenever growth is initiated, but spores are not formed in large numbers (*i.e.*, over 3 per cent) at pH 6.1, or below, in four days. An optimum pH for spore formation lies be-

tween 6.9 and 7.4. The range of optimum pH for sporulation is the same as that for growth and for germination of the spores.

The work of Knaysi (91) with strain C<sub>2</sub> of *B. mycoides* showed that endospores may be formed within a wide interval of pH, but that there is a definite optimum between pH 6.6 and 6.8. "As the pH increases, its unfavorable effect on sporulation quickly becomes noticeable. The number of endospores drops sharply as one passes the neutral point, although the total population may not be materially reduced."

3. *Nutrilites*. Williams (169) was unable to get satisfactory spore yields with *B. subtilis* in several synthetic media, whereas Roberts (144) reported 60 to 70 per cent endospores of the same species, within 5 days, in a relatively simple synthetic medium, compared to less than 30 per cent in 1 per cent peptone solution. Roberts and Baldwin (146) found that "A greater percentage of *Bacillus subtilis* cells sporulate in Bacto-peptone broth treated with charcoal than in untreated peptone water. . . . It is suggested that spore formation in Bacto-peptone water is inversely proportional either to some particular food factor or factors or to some factor directly inhibitory to the spore-forming process." Witte and proteose peptones did not respond to this treatment. Hayward (71) observed no beneficial effect on the sporulation of *B. subtilis* from the addition of thiamine, nicotinic acid, pantothenic acid, riboflavin, pyridoxine, or biotin to vitamin-free casein hydrolyzate; a slight stimulation was attributed to inositol.

Knaysi (91) found that, with strain C<sub>2</sub> of *B. mycoides*, "Vitamin B<sub>1</sub> promotes sporulation (but does not seem to affect growth) on agar slants of a simple medium in which there is tendency for acid by-products to accumulate; it has no effect on either growth or sporulation in agar slants of vitamin-free casein hydrolyzate in which no acid by-products accumulate. The effect of the vitamin in the synthetic medium is indirect and due to an increase in the rate of decarboxylation of the acid by-products. It is assumed that the vitamin is readily synthesized in the casein hydrolyzate medium."

Dutky (49) recently reported that he developed several media for the growth of *Bacillus popilliae* and *B. lentimorbus*. These media "gave consistently high yields of vegetative forms, but none was adequate for sporulation." Whether or not this means that sporulation requires an additional growth factor is not clear.

4. *Desiccation of the medium*. A relationship between loss of water from the environment and sporulation has long been considered. According to Soyka (159) sporulation in *B. anthracis* and *B. subtilis* is hastened by the addition of soil, and there is an optimum proportion of moisture to soil. Migula (125) stated that "Moisture is as necessary for sporulation as it is for growth. Sudden drying hinders sporulation chiefly because it stops all development in the bacteria. On the other hand, gradual drying seems to promote sporulation possibly . . . as a result of concentration of the nutrient medium." (Translated by the reviewer.) Holzmüller (76) observed that sporulation is hastened when material containing aerobic, sporeforming bacteria is allowed to dry on a cover glass.

Darányi (32, 33) attributed sporulation exclusively to drying of the colloids which make up the cells. "This colloid dehydration may arise 1. through the aging of the bacteria or 2. with young bacilli too by artificial withdrawal of water. Dehydration was carried out by alcohol vapor, calcium chloride etc. and also by the drying of bacilli on a glass surface in an incubator during two days, the last method proving the most satisfactory. With cultivation of so dried virulent anthrax bacilli we can produce 90 per cent spores in so short a time as 18 hours. Other experiments with non-virulent anthrax bacilli, *B. subtilis*, *B. anthracoides*, etc., also support this statement. The aging is also a natural loss of water, known as Histeresis of the colloids." Exhaustion of the medium is believed to act through dehydration of the cell colloids. Thus, dehydration seems to predispose bacteria to sporulate even when they are subsequently cultivated in a normal medium. The work of Darányi was reported with favorable comment by several subsequent investigators who, so far as the reviewer knows, had not repeated his experiments.

More recently, the effect of a gradual drying of the medium on sporulation, in strain C<sub>2</sub> of *B. mycoides*, was investigated by Knaysi (91). Agar slant cultures were incubated in an atmosphere where the moisture content was regulated with solutions of NaOH and H<sub>2</sub>SO<sub>4</sub>. "The atmosphere was sufficiently large to eliminate oxygen as a factor. . . . Comparison with similar slant cultures plugged with cotton convinced us that the latter precaution was effective. . . . The data . . . show that, under the conditions of our experiments, drying exerts a harmful effect. . . . Our data show, however, that drying is not usually of much importance as a factor in the formation of endospores by strain C<sub>2</sub> of *Bacillus mycoides*."

5. *Miscellaneous factors.* a. *Light.* Schreiber (155) exposed to direct sunlight cultures in the stage of active growth. The effect of heat was eliminated by partial immersion of the tubes in running water. After an exposure of 15 minutes, *B. anthracis* was unable to form spores; the same effect was induced in *B. tumescens* by an exposure of 40 minutes, and in *B. subtilis* by an exposure of 1 hour. Before the vegetative cells die, their content becomes granular, their protoplasm contracts, and involution forms appear.

Holzmüller (76) exposed agar plates inoculated with spores of *B. mycoides*, or one of several related species, to direct sunlight. The plates were sealed with paraffin and immersed in running water at 26 C to eliminate the effect of heat. *B. mycoides*  $\delta$  germinated in 24 hours; *B. mycoides*  $\alpha$  in 40 hours; *B. mycoides*  $\beta$  and *B. effusus* in 48 hours; *B. olfactorius* in 60 hours, and *B. nanus* in 5 days. *B. mycoides* Flüggé and  $\gamma$ , and *B. dendroides* did not germinate. After 8 days in the sun (?) *B. mycoides*  $\alpha$  and  $\delta$  had formed normal colonies, viable spores, and involution forms; strain  $\beta$  did not grow after germination; most of the cells had died or changed into involution forms. *B. olfactorius* grew very well and formed many spores but no involution forms. *B. effusus* and *B. nanus* grew fast, forming involution forms but no spores. Cultures of the species which, after 8 days in the sun, failed to germinate were placed in the dark at 30 C; *B. dendroides* germinated in 30 to 36 hours and grew normally; *B. mycoides* Flüggé and  $\gamma$  did

not grow. It is interesting to note that all five strains of *B. mycoides* behaved differently toward sunlight.

*b. Stage of the culture.* It was thought by some of the early investigators that the formation of endospores is preceded by a definite number of cell divisions which varies with the species. In discussing this question, Migula (125) wrote: "In *Bacillus subtilis*, the formation of spores takes place when numerous cells, which develop from one or a few spores, grow into long threads each consisting of many cells. The number of vegetative divisions is very great. In *Bacillus sessilis*, according to Klein. . . , spore formation takes place, under similar conditions, after only a few divisions (about 8). However, one can prevent sporulation in both species when one transfers the culture, before sporulation, into a fresh medium. Thus, in this case, an external factor, the transfer to a fresh medium, hinders sporulation. Therefore, sporulation must not necessarily follow a definite number of divisions. When one observes in a moist chamber a droplet of bouillon containing a few spores of *Bacillus subtilis*, one finally observes numerous cells, or threads, which developed from the few spores. In another droplet of bouillon which contained a hundred times more spores than the first, the number of cells at the end of vegetative growth, when spores are formed, is not substantially larger than in the preparation inoculated with a few spores. Spore formation takes place when for a definite amount of nutrients a definite number of cells have been formed, regardless of the number of spores inoculated. Consequently, spore formation takes place when the medium becomes unsuitable for vegetative growth." (Translated by the reviewer.)

These simple experiments performed by Migula showed clearly that for any given organism the number of vegetative cells formed in a culture before spore formation depends on the amount of available food in the medium. However, they did not settle the question of whether or not the formation of spores in a given culture begins at a definite stage of development of that culture.

Studying *B. megatherium*, Henrici (73) wrote: "It will be noted . . . that spore formation commenced practically at the point of inflection between the logarithmic growth phase and the resting phase . . . . Since spore formation is a characteristic of the resting phase, the rate of spore formation should be influenced by those factors which also tend to influence the rate of growth and the form of the growth curve." From his study of *B. cohaerens*, which was reviewed in a previous section, he concluded: "It would appear, therefore, that the rate of spore formation is determined not by the concentration of cells alone, but rather by the density of the population in relation to concentration of food-stuff in the medium."

The observations of Greene (68) on sporulation within the colony may be recorded under any of several headings. In *Clostridium acetobutylicum*, sub-surface colonies sporulate principally at the periphery; in surface colonies sporulation is confined to the central and basal portions. In *C. pasteurianum*, surface colonies do not show a regular pattern of sporulation. In old colonies, however, one observes a transition from vegetative cells at the margins to mature spores in the middle; the intermediate zones contain young sporangia. In *B.*



*acetoethylicum*, subsurface colonies sporulate only at the periphery. Surface colonies tend to sporulate at the surface when the medium contains a relatively high agar concentration and a relatively low glucose concentration. When the medium contains an excess of  $\text{CaCO}_3$ , sporulation tends to be profuse.

c. *Presence of other bacteria.* It was noted by Brefeld (10) that sporulation in *B. subtilis* fails or is strongly hindered in mixed cultures.

Simonds (157, 158) observed that "In the mixed fecal flora, the bacillus welchii may sporulate even in the presence of free acid or fermentable carbohydrate . . . . That this ability to sporulate in the presence of free acid or fermentable sugar depends upon symbiotic relations of the bacillus welchii with one or more species other than the colon bacillus, is indicated by the negative results obtained when a pure culture of the bacillus welchii was grown in symbiosis with a pure culture of the colon bacillus."

*Discussion.* The literature reviewed above shows that the formation of endospores by certain bacteria is a normal process, highly sensitive to the environment. A number of those who investigated the effect of the environment attempted to answer the question: What is it that induces bacteria to form endospores in an environment favorable for that process? In a discussion of this question, Knaysi (91) wrote: "In studying the environmental factors which affect the formation of endospores, the various investigators have dealt with each factor as an independent variable. In reality, those factors are so interrelated that the proper evaluation of any one factor is not possible unless its relation to other factors is elucidated. This requires familiarity with the physiological behavior of the organism investigated. In the case of strain  $C_2$  of *B. mycoides*, and indeed of a number of other members of the genus *Bacillus* with which we are familiar, it is important to know that the temperature, the concentration of oxygen, the state and density of the population, the utilization of nutrients, the accumulation of the by-products of metabolism, the spontaneously developed pH, etc., are intimately interrelated. In the presence of a fermentable sugar, the organism can grow anaerobically, but the acid by-products of glycolysis, chiefly lactic acid, accumulate and the pH drops to a minimum which varies with the medium; under strictly anaerobic conditions, endospores are not formed. Now what is the limiting factor? Is it the absence of oxygen or is it the accumulation of the acid by-products? When oxygen is plentiful, oxidation is usually immediate and complete; the pH drops only slightly and then rises to a maximum. Under these conditions endospores are formed, but only as the alkalinity of the culture has considerably risen. Now what caused the formation of endospores? Is it the presence of oxygen, or the alkalinity, or the exhaustion of the nutrients? Spores are formed best on the surface of a solid medium. Is it because of greater availability of oxygen or is it because of gradual drying? All those questions must be answered before a clear picture of the conditions necessary, helpful, or detrimental for sporulation are clearly known."

Knaysi then proceeds to analyze his own data, following which he further writes: "The only possible conclusion to be drawn from this work with strain

C<sub>2</sub> of *Bacillus mycoides* is that endospores are formed in an aerobic environment by healthy cells facing starvation. . . . In drawing this obvious conclusion, we do not wish to be accused of teleologic tendencies which are detrimental to a final solution of the mechanism of sporulation. To say that cells starved in the presence of oxygen proceed to form endospores is to acknowledge a biological fact. Why the depletion of nutrients induces the cell to sporulate is a question yet to be answered. At the present, one can merely speculate."

The conclusion that an endospore is formed by a healthy cell facing starvation is undoubtedly true for all strongly aerobic members of *Bacillus*; it is also likely to be true, after due consideration of physiological peculiarities, for other members of this genus as well as for *Clostridium*. Although there is a paucity of data about sporulation in anaerobic organisms, available information indicates that it is not governed by a law fundamentally different from the one that governs sporulation in *B. mycoides*.

### Germination

When a viable endospore is placed in a suitable environment, it proceeds to germinate. For any one spore, germination is a gradual process which is arbitrarily considered to begin as the spore begins to swell and lose in refringence and, when the spore sheds its coat, to end as the germ cell emerges from the coat. When the spore "absorbs its coat," it is very difficult to set even an arbitrary limit for the termination of the process. Swann (161), studying *B. anthracis*, defined the period of germination as the time which elapses between the commencement of incubation and the first division of the germ cell. The time it takes a spore to germinate varies considerably with the strain and with the environment. Spores of a given strain, however, do not all germinate simultaneously in the same environment, and the germination period for the strain is a statistical figure. When the spores are not unusually old and have not suffered injury, the differences between germination periods of any two spores of the majority is much smaller than the germination period of either. Nevertheless, there usually are in every culture spores which may take several times the length of the average germination period. Swann (161) studied the effect of age on spore germination in *B. anthracis*. He found that age, up to 47 days, has no effect on the germination period, provided the spores are kept moist. Spores from dried cultures, however, required much longer time. Swann considered dead any spore of *B. anthracis* which did not germinate in 7 days. On this basis, he concluded that "about 5 per cent of young anthrax spores (2 to 3 days old) and about 55 per cent of old dried spores (1 year old) are dead." Swann was apparently unaware of the work done, chiefly in this country, in relation to delayed germination or "dormancy" of endospores.

Delayed germination of spores subjected to sublethal heat was observed by Miquel and Lattraye (126) and by Eckelmann (51). Burke (16), Dickson *et al.* (44, 45) and Dickson (43), recorded long periods of dormancy in heated spores of *Clostridium botulinum*. Burke (17) observed dormancy in unheated spores of *C. botulinum*; Burke *et al.* (18), in unheated spores of *B. subtilis* and *B.*

*megatherium*. It was believed that dormancy is a natural phenomenon inherent in the spore, and that this "normal" tendency is intensified by injury from heat. Morrison and Rettger (128, 129) made a comprehensive study of this phenomenon in several members of *Bacillus* and concluded "that the dormancy of aerobic bacterial spores is largely, if not entirely, determined by conditions in the environment of the spores, and that these factors must be taken into consideration, perhaps specifically for each species, before so-called 'inherent' or 'normal' dormancy of bacterial spores can be established." The reviewer's sympathy with this interpretation will be shown below, in the discussion. See also (172) and (29).

The environmental factors investigated in relation to spore germination are: temperature; composition of the medium (not including oxygen); molecular oxygen; pH, osmotic pressure, and surface tension of the medium; light.

1. *Temperature.* a. *Temperature of incubation.* Figures on germination periods of many spore-forming bacteria at various temperatures may be found in a number of the papers referred to in this review. These figures vary with the organism and temperature from about an hour to days. In most of the common members of *Bacillus*, the majority of the spores of a culture germinate, near the optimum temperature, within a time interval of 1 to 4 hours.

Of considerable interest is the relation of the cardinal points of germination to those of growth and spore formation. Of the 28 members of *Bacillus* studied by Blau (9), 22 had the same maxima for both spore germination and growth, four (*B. alvei*, *B. carotearum*, *B. robur*, and *B. simplex*) had higher maxima for growth than for germination, and only two (*B. asteroides* and *B. sphaericus*) had higher maxima for germination than for growth. Holzmüller (76) concluded, from a study of 5 strains of *B. mycoides* and 4 other related species, that, for any given strain, the optima for germination and for growth were the same, but that the temperature range for germination was narrower than that for growth.

b. *Exposure to "sublethal" temperature.* When a spore-containing culture is exposed, for a short period, to a temperature above "that" which kills the vegetative cells and below "that" which kills the spores and then used to inoculate a fresh medium, the subculture is usually endowed with considerable vigor. This is the basis for the practice of "heat-shocking" cultures which had deteriorated in certain respects because of frequent transferring or other growth conditions. The effect has been attributed to the elimination of vegetative cells among which may be undesirable or weak variants. Although this is probably one of the chief results of the procedure, the work of Evans and Curran (60) and of Curran and Evans (31) shows that the spores themselves may be affected by the treatment. With *B. megatherium*, *B. cereus*, *B. subtilis*, *B. cohaerens*, *B. fusiformis*, and other unidentified mesophilic strains, these authors found that exposure of the spores to 85 C for 8 to 10 minutes accelerated their germination. The effect, however, depended on the nature of the medium in which the spores were heated and incubated, and on the pH of the medium in which they were heated. Heating in distilled water and incubating in glucose broth accelerated

germination in 5 out of 9 cultures; heating and incubating in glucose broth activated seven; heating and incubating in evaporated milk activated all 9 cultures. Spores heated and held in distilled water retained their altered capacity for germination after one week. Similar observations were made on 12 thermotolerant or thermophilic cultures of *Bacillus*. Without heat treatment, a large proportion of the potentially viable spores did not germinate. NaCl (0.5 per cent) had a depressing effect.

2. *Composition of the medium.* Cohn (23) observed that the spores of *B. subtilis* did not germinate in the medium where they were formed, but only when transferred to a fresh medium. A similar observation was made by Koch (104) on *B. anthracis*. On the other hand, Preisz (142) attributed the formation of secondary colonies by *B. anthracis* to germination of spores and subsequent growth of the vegetative cells. The recent work of Knaysi (91, 92) with strain C<sub>2</sub> of *B. mycoides* showed that the limiting factor for the germination of endospores in their mother culture is food. When traces of tryptone or broth are added to a culture which already sporulated, germination follows. In slant cultures, germination may be due to the diffusion of nutrients from remote parts of the slant; in aerated, liquid cultures, it may be induced by increasing the rate of aeration. Knaysi, Baker, and Hillier (99) considered the transparency of certain spores in their mother cultures as evidence of incipient germination.

a. *Kind of nutrients.* It was stated by Holzmüller (76) that the spores of several strains of *B. mycoides* and related organisms do not germinate in distilled water or in physiological NaCl solution. Knaysi (92) confirmed this with strain C<sub>2</sub> of *B. mycoides* and further found that the washed spores of this organism do not germinate in the following solutions: potassium phosphate; potassium nitrate; lactose; potassium phosphate and nitrate; potassium phosphate and nitrate + lactose. Lactose is not utilized by this organism. On the other hand, the spores "are able to germinate normally in a solution of glucose . . . without the addition of a source of nitrogen. When this glucose solution is buffered with potassium phosphate . . . at a pH of about 7, germination is followed by growth and sporulation . . . . It is concluded that endospores of the strain investigated contain relatively large amounts of a nitrogen-containing reserve material not suitable as a source of energy, and that they contain no other reserve material for that purpose" (*i.e.*, for energy). Knaysi and Baker (98) made use of these observations to obtain vegetative cells of *B. mycoides* transparent to electrons of moderate velocities and found that the source of nitrogen present in the spore has the physiological and staining properties of ribonucleic acid.

When the spore suspension of strain C<sub>2</sub> of *B. mycoides* is not much more than two weeks old at room temperature (25 to 27 C) and was prepared from slant cultures containing meat infusion and glucose and not much more than one week old at room temperature, many spores germinate and the germ cells grow and multiply in a solution of glucose and sodium acetate without the addition of other mineral salts or of metabolites to the solution.

*b. Concentration of nutrients.* Since the endospores of *B. mycoides*, C<sub>1</sub>, do not germinate in distilled water or in solutions of various salts but do germinate, and the vegetative cells grow and form spores on bacto agar gel, prepared with distilled water and to which no nutrients were added (92), there must be a minimum concentration of food below which germination does not take place. Obviously, this minimum must be very low. Since the spore contains a source of nitrogen and phosphorus, germination is possible without the addition of extraneous sources of these elements. The minimum for the source of energy has not been determined but is, doubtless, below 0.1 per cent of glucose. In solutions of peptone, which is both a source of nitrogen and a source of energy, the minimum concentration is, according to Curran (26), 0.025 per cent.

The effect of increasing the concentration of food seems to depend on the osmotic pressure (76). Curran (26) observed considerable retardation of germination in 10 per cent peptone solution compared to 1 per cent solution. The effect of substances which do not materially affect the osmotic pressure has not been studied. It is possible that high concentrations of such substances will also cause inhibition by reducing the amount of available moisture.

*3. Molecular oxygen.* Holzmüller (76) stated that the oxygen of the air has no effect on the spore germination of facultative bacteria. Leifson (113) determined the maximum pressures of molecular oxygen which allow growth of several common members of the genus *Clostridium*, and the minimum pressures which allow the growth of four common members of *Bacillus*. *C. botulinum*, *C. tetani*, *C. novyi*, *C. oedematiens*, and *C. septicum* were inhibited by an oxygen pressure equivalent to 2 cm of mercury. *C. chauwei* and *C. sporogenes* grew at that pressure. *B. anthracis* and *B. cereus* did not grow below 2 cm; *B. vulgatus* was practically inhibited by 1 cm; *B. circulans* grew without molecular oxygen. In this work, there is no direct reference to germination. Knaysi and Dutky (100) inhibited the growth of a strain belonging to the group of *C. butylicum* by an air pressure equivalent to 270 to 290 mm of mercury in broth containing meat extract, peptone, and glucose; here also, no reference is made to germination. Knaysi and Gunsalus (101) showed that the Marburg strain and the Ford and Lawrence strain of *B. subtilis* grew anaerobically in the presence of a source of energy that can be utilized anaerobically, such as a fermentable sugar.

*4. Concentration of hydrogen ions.* It was observed by Knaysi (92) that washed endospores of *B. mycoides*, C<sub>2</sub>, incubated in a solution of glucose, or of glucose and potassium nitrate, have a tendency to germinate. "However, typical germination is observed only until the pH drops to a value somewhere between 5 and 6. Below this pH, the vegetative cells already formed are unable to survive, and typical germination is inhibited probably because of the destruction of potential cells in the early stages of germination. Finally, the cellular elements in such suspensions consist of a reduced number of unchanged spores with pairs or clumps of various sizes in which some still refractive, but apparently shrunken endospores are found with nonrefractive spores, shells and shell fragments; occasionally, a refractive body may be seen attached to a cracked exine." The minimum pH reached when endospores are suspended in

an unbuffered solution of glucose is 4.8; in a solution of glucose and potassium nitrate the pH reaches 4.0 or slightly below. In the latter solution, a suspension of vegetative cells reaches a minimum pH of only 5.0 to 5.2. "The difference must be sought not only in the low permeability of the exine, but also possibly in a greater concentration of nondiffusible substances in the endospore, as would be expected from a Donnan equilibrium. In any case, it is probable that the internal pH of the vegetative cell in the solution of glucose +  $\text{KNO}_3$  at pH 5 is about the same as that of the endospore in a similar solution at pH 4.0. The value of that internal pH is of considerable importance and should correspond to the level of acidity at which the enzymes which take part in the fermentation of glucose are inhibited."

5. *Osmotic pressure of the medium.* Holzmüller (76) studied the effect of osmotic pressure of the medium on the spore germination of five strains of *B. mycoides* and four related species, and concluded that there is an optimum osmotic pressure which varies with the strain. When the osmotic pressure of the medium is below the optimum, the addition of sodium chloride enhances germination, when it is above the optimum, it retards it. Holzmüller also concluded that the osmotic pressure of a nutrient medium is more important for germination than the kinds of nutrients it contains. The effect of the osmotic pressure on growth is different from its effect on germination. In a medium of low osmotic pressure, growth is not enhanced by the addition of sodium chloride. Furthermore, growth may be very slow in a medium of optimum osmotic pressure for germination.

Eijkman (55) determined the minimum inhibiting concentrations of a number of substances on the spore germination of *B. anthracis*. On the basis of the behavior of these substances, they were divided into 3 groups. The first group (e.g.,  $\text{NaCl}$ ,  $\text{KNO}_3$ , glucose) owe their inhibiting action to their osmotic pressure; the inhibiting pressure corresponds to a depression of the freezing point of about 3.5 C. The second group (e.g.,  $\text{NH}_4\text{Cl}$ ,  $\text{Na}_2\text{HPO}_4$ ) inhibit germination at a pressure below that of the first group; therefore, their action is due only partly to osmotic pressure; the third group (urea, methyl and ethyl alcohol) exert no osmotic effect. Eijkman attributes the behavior of the third group to permeability and suggests that the endospore may not be as isolated from its environment as usually presumed.

More recently, the effect of osmotic pressure on germination of the spores of *B. mycoides* was carefully investigated by Curran (26). His observations are summarized as follows: "1. Germination of spores is most rapid and complete at relatively low osmotic pressure. 2. The osmotic tension necessary for optimum germination is definitely lower than that required for maximum vegetative development. 3. The exact osmotic pressure at which optimum germination occurs depends upon the nature of the nutrient material, the more suitable or readily available the food the lower the osmotic pressure at which optimum germination occurs. 4. There is a definite minimum concentration of food below which no germination occurs, irrespective of the osmotic pressure . . . 5. A deficiency of food is probably the limiting factor in most low pressure

solutions. 6. In these experiments there was no evidence to indicate a purely osmotic limitation in low pressure solutions. 7. When the minimum nutritional requirements for optimum germination are exceeded, an increased concentration of food may retard the rate of germination due to the unfavorable osmotic pressure created by the added nutrients. Under such conditions a certain degree of dilution will accelerate the rate of germination. Conversely, increasing the food supply increases the rate of germination only when the nutrient concentration is below the critical minimum. 8. There is a rather definite limiting osmotic pressure for germination. This corresponds roughly to 36-46 atmospheres . . . ”.

6. *Surface tension.* According to Curran (27), surface tension is not a significant factor in the germination of the spores of *B. mycoides*. A reduction in surface tension from 50 to 43.4 dynes had no appreciable effect upon either the rate of germination or the total percentage of viable spores. At 35.1 dynes, the rate of germination was slightly retarded and the total percentage of spores which germinated within 5 hours was slightly reduced. At 30.7 dynes, about 50 per cent of the spores were able to germinate within 6 hours. Curran concluded: “Depression of the surface tension can not be relied upon to prevent or accelerate spore germination under ordinary conditions.”

7. *Light.* Holzmüller (76) stated that diffuse light has no effect on germination, growth, or sporulation. The effect of direct sunlight was reviewed in the section on the relation of the environment to endospore formation.

8. *Oxidation-Reduction Potential.* The relation of the oxidation-reduction potential of the medium to the growth of anaerobic bacteria was first pointed out by Clark (21a) and subsequently confirmed and extended to other bacteria by many investigators (21c, 47a, 1a, b, 100, 99a, 172a, 130a, and others). The effect of potential is most obvious in the initiation of growth of small inocula, or when the cells had sustained certain injury, or when the bacteria are inoculated into unusual media. However, inoculation into a medium of a suitable potential, all other factors being the same, always results in faster and more vigorous growth in the early stages of the culture. Oxygen plays a double rôle, the first specific, the second nonspecific by affecting the potential of the medium. These two rôles have often been confused.

Since the germination of spores involves growth, it should be affected by the oxidation-reduction potential of the medium. Indeed, Fildes (61a) showed that the spores of *Clostridium tetani* germinate only when the potential of the medium is below a certain value. Recently, Knaysi (97a) observed that germination of the spores of *Bacillus mycoides* in a solution of glucose and sodium acetate is favored when the solution is freshly autoclaved or when traces of phosphate are added. The effect of autoclaving and of the phosphate on germination was related to their effect on the potential of the solution. The beneficial effect on germination of preheating spores to sublethal temperatures (60, 31) can also be readily related to the influence of heat on the potential of the medium and on the internal potential of the spores.

*Discussion.* Normal germination of the endospore is a process involving

growth, and is affected by factors which affect growth. Indeed, germination is growth in a special environment rich in nitrogen supply and partly protected from the external medium usually by a double coat. Any genuine difference between growth and germination must be attributed to that circumstance. For instance, germination may take place below the limiting pH for growth because it is unlikely that a given pH of the external medium corresponds to the same pH within the spore and within the vegetative cell (92); or germination may appear to have less nutritional requirements than growth of the vegetative cell because the required substances may be present in the spore and are not easily lost by washing because of the low permeability of the spore coats. It must be emphasized, however, that during germination the spore acquires vegetative characteristics and, if external conditions are inimical to growth, germination may never be completed or, if completed, the germ cell may perish.

The question of whether or not there are spores which remain dormant for prolonged periods in an environment suitable for germination needs further consideration. Without wishing to deny the theoretical possibility of the existence of rare spores completely impermeable to food material, or genetically defective, the reviewer finds no sound, experimental basis for the phenomenon of dormancy. Long and careful study of the minimum requirements for spore germination in strain C<sub>2</sub> of *B. mycoides*, in connection with the development of vegetative cells transparent to electrons, led the reviewer to the conclusion that normal, mature spores of this organism are able to germinate in a solution of glucose and sodium acetate; and that this ability is enhanced when the sugar is activated by heat or by traces of potassium phosphate. When the spores are allowed to age, in culture or in aqueous suspension, their ability to germinate is decreased and they gradually fail to respond to activation of the sugar by heat or, later, by phosphate. These spores are "dormant" in the glucose, acetate solution, for the addition of a small quantity of yeast extract, or of tryptone, induces many of them to germinate. Indeed, it is possible to raise, at will, the percentage of germinating spores by controlling the composition and the reduction potential of the medium. It seems that the normal, mature spore contains all that is needed to germinate other than a source of energy. They contain enzymes (52, 53, 152, 24, 163, 167), a source of nitrogen and phosphorus (92, 98), and various minerals such as Ca, K, Cu, and Mn (28). It seems that aging involves leakage or destruction of a substance, or substances, necessary for growth, and which must be supplied in the medium. The poor germinability of dried spores is probably due to destruction by oxidation. The "dormancy" of some heated spores is largely due to leakage because of an increased permeability, since heated spores, according to Effront (53), are enzymatically more active the more difficult their germination. Thus the reviewer is inclined to agree with Morrison and Rettger (129) who wrote: "Experimental evidence, is presented to show that the dormancy of aerobic bacterial spores is largely if not entirely, determined by conditions in the environment of the spores," and with Curran and Evans (29) that "bacterial spores which survive drastic killing influences are much more exacting in their nutritive requirements than



the less resistant individuals which comprise the bulk of the viable population before treatment."

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# CYTOCHEMICAL INTERPRETATION OF THE MECHANISM OF PENICILLIN ACTION<sup>1</sup>

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"Among the lower beings, even more than among the higher  
animals and plant species, life destroys life."

Pasteur and Joubert (1877)

According to current usage, the term antibiotic designates a product of the metabolism of one microorganism that is antagonistic to the continuation of the normal life activities of another microorganism when present even in very low concentrations. Since the early literature on antibiosis has been covered extensively (4, 7, 29, 70, 71, 145, 190), we shall dispense with this phase of the subject.

The penicillins owe their outstanding therapeutic position to the fact that they exert a strong selective action against certain types of bacteria in concentrations far below those required to produce appreciable effects on animal tissues. They may be considered virtually non-toxic. The activity, A, of an antiseptic may be expressed in terms of the maximum volume (in ml) of broth in which 1 gram of compound will inhibit the growth of a test organism; and the toxicity, T, may be expressed as the maximum weight (in g) of some suitable laboratory animal that can be killed by injection of a like quantity of the same compound. The ratio, A/T, may be used as an index of relative activity and toxicity. According to this system, a large number indicates relatively low toxicity and a small number relatively high toxicity in comparison with activity. Using *Staphylococcus aureus* as the test pathogen and adult mice (20 g each, injected intraperitoneally) as test animals, the A/T ratio has been found to be 3 for anemonin, a very toxic compound, 100 for sulfathiazole, and greater than 100,000 for penicillin (28).

The virtual lack of toxicity of penicillin was recognized at an early date when it was shown in 1944 that any toxicity of purified penicillin salts could very likely be attributed to the cation. It was found that the toxicity of the sodium, ammonium, strontium, calcium, magnesium and potassium salts of penicillin ranked in the same order as that of the acetates, which have a non-toxic anion (198).

The only unfavorable physiological effects that have been reported, apart from the urticaria that occasionally develops in sensitive individuals, are delayed clotting of blood (69a, 123), the precipitation of convulsions following intrathecal injection (192), and partial inhibition of phagocytosis (197). With regard to the first of these observations, however, it should be pointed out that a contradictory report has been published, i.e., that penicillin accelerates clotting

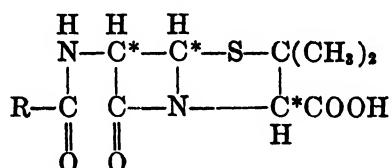
<sup>1</sup> This work was supported, in part, by a generous research grant from the Cutter Laboratories, Berkeley, California.

(131). The discrepancy in observations may be due to the widely different concentrations of penicillin that were employed. The other reported untoward effects have been observed only with large doses, far in excess of those administered clinically.

The penicillins are further characterized by the fact that they inhibit susceptible bacteria more effectively both *in vivo* and *in vitro* when the environment is most favorable for growth and by the fact that within wide limits their activity is not affected by the number of bacteria present (16) whereas many other chemotherapeutic agents are less effective if contamination is heavy.

#### SOME IMPORTANT CHARACTERISTICS OF THE PENICILLINS

The penicillins comprise a family of antibiotics that are biosynthesized by several species of molds, notably penicillia belonging to the notatum-chrysogenum group. More than a dozen penicillins are recognized to occur naturally and from some of these (principally penicillin X) many derivatives have been prepared. However, only five penicillins are commonly encountered, all possessing the same fundamental chemical structure:



They are characterized stereochemically by the three asymmetric carbons (starred in the above formula) which are responsible for the optical rotation of their solutions. The several penicillins differ markedly in their pK values, their distribution between polar and non-polar solvents, and their biological activity *in vitro* and *in vivo* according to the character of the R group (table 1).

Laboratory experiments with animals, and clinical trials and evaluations have shown that the relative effectiveness of the different penicillins *in vivo* depends upon the rate of excretion, degree of protein-binding in the blood, the pathogen involved, and several other factors. In practice, benzyl penicillin (penicillin G) has been found to be the most generally useful (38, 59, 60, 61, 99, 101, 166).

In this paper, following established custom, the term penicillin will be used to designate a salt of benzyl penicillin unless otherwise specified. An average effective therapeutic blood level of penicillin for systemic treatment of many infections is considered to be about 0.075 unit per ml of serum. Since one unit of the pure sodium salt of benzyl penicillin weighs 0.6 microgram, an average effective therapeutic serum level represents a dilution of 1 part in approximately 22 million. For infections due to highly sensitive organisms, serum levels of 0.03 units per ml (1 part penicillin in approximately 55 million) often are adequate. Solutions containing 0.1 unit penicillin per ml, or 1 part in approximately 16.6 million, have been shown to be lethal *in vitro* to cells of average strains of *Staphylococcus aureus*. These concentrations are far below the effective antibacterial concentrations of other antibiotics and of the commonly employed

antiseptics. A comparison of the *in vitro* activity of penicillin with some other antibiotics is shown in table 2. It should be pointed out, however, that the figures are based on agar plate assays and that the results obtained by this technique depend not only on biological phenomena, but also on the physical phenomenon of diffusion. Consequently, since the molecules of some of the compounds may not diffuse as readily as those of others, the exact order of activity for some of the compounds is different in serial dilution tests, although by this test also penicillin ranks first in effectiveness.

As has been pointed out by Dubos (51, 52, 53) and others, the fact that penicillin exerts a strong selective action in relatively low concentrations against many types of bacterial cells without manifesting any appreciable toxicity toward other living structures indicates that it does not owe its action to a general or non-specific protoplasmic poisoning as do many cell poisons such as the halogens, salts of heavy metals, phenols, etc., but suggests rather, that its

TABLE 1  
*Characteristics of five common penicillins\**

R GROUP		COMMON NAME	MOL. WT. OF Na SALT	pK	IN VITRO ACTIVITY <sup>b</sup> (UNITS/MG) AGAINST	
Carboxyl residue	Acetamide linkage				<i>S. aureus</i>	<i>B. subtilis</i>
phenylacetic	benzyl	G	356.2	2.5	1,667	1,667
<i>p</i> -hydroxy-phenyl- acetic	<i>p</i> -hydroxy-benzyl	X	372.2	2.5	900	1,500
3-hexenoic (hydro- sorbic)	$\Delta^2$ pentenyl	F	334.2	2.7	1,500	970
caproic	<i>n</i> -amyl	dihydro F	335.2	2.7-	?	?
octanoic (caprylic)	<i>n</i> -heptyl	K	364.3	5.0	2,300	700

\* Compiled from data of Schmidt, Ward and Coghill (165), Goodall and Levi (86, 87), Henry and Housewright (95), and unpublished data from the Cutter Laboratories.

<sup>b</sup> Determined by standard 16 hour cylinder-plate assays.

effect is highly specific. Evidence to support this idea is provided by the facts that microorganisms are most susceptible to the action of penicillin when they are in the logarithmic phase of growth and that incorporation in the media of growth stimulating substances enhances the action of penicillin *in vitro* (17, 35, 100, 102, 105, 113, 128, 154). Thus when the cells are dividing most rapidly, and are exhibiting the greatest need for oxygen, they are most sensitive to the action of penicillin. Conversely, conditions (such as low temperature) which decrease the rate of division, tend to decrease the susceptibility of bacteria to the killing power of penicillin (35, 103, 111). In this connection it is interesting to note that strains of staphylococci that are resistant to penicillin have been reported to be characterized by depressed growth rates (72, 75).

Strains of organisms that become resistant to one type of penicillin become correspondingly resistant to the others (62) as would be predicted, since penicillin-fastness depends upon the slowing down of metabolic rates. It should be

noted, however, that acquired "fastness" to penicillin is only temporary. The organisms return, after several generations, to their normal rates of growth and become susceptible again to the action of penicillin (184).

#### AMORPHOUS VS. CRYSTALLINE PENICILLIN

The earlier reports on the mechanism of penicillin action were based on studies with impure penicillins. Among the impurities that may be found in such

TABLE 2

*Parts per million of antibiotics required to produce inhibition zones 20 mm in diameter on standard assay plates seeded with Staphylococcus aureus<sup>a</sup>*

ANTIBIOTIC	PPM	AUTHORITY
1. Penicillin	1	Abraham and Chain (1)
2. Helvolic acid	6	Chain <i>et al.</i> (34)
3. Mycophenolic acid	31	Clutterbuck and Raistrick (37) Florey <i>et al.</i> (73)
4. Protoactinomycin	200	Gardner and Chain (81)
5. Citrinin	62	Tauber <i>et al.</i> (180) Heatherington and Raistrick (94)
6. Gliotoxin	31	Weindling (195) Johnson <i>et al.</i> (107)
7. Puberolic acid	200	Oxford <i>et al.</i> (142)
8. Fumigatin	1,000	Anslow and Raistrick (5) Oxford and Raistrick (140)
9. Spinulosin	4,000	Birkinshaw and Raistrick (21) Anslow and Raistrick (6)
10. Anhydro-3-hydroxy-methylene tetrahydropyrone-2-carboxylic acid <sup>b</sup>	100	Chain <i>et al.</i> (33a)
11. Aspergillie acid	1,000	White and Hill (201) Menzel <i>et al.</i> (125)
12. Penicillic acid	1,000	Birkinshaw <i>et al.</i> (19) Oxford <i>et al.</i> (141)
13. Kojic acid	6,666	Birkinshaw <i>et al.</i> (18, 20) Jennings and Williams (106)
14. Streptomycin	500	Schatz <i>et al.</i> (163) Schenck and Spielman (164)

<sup>a</sup> Calculated and rearranged from data compiled by Heatly and Philpot (93).

<sup>b</sup> Various named expansin, clavatin, clavacin, claviformin, patulin, penicidin.

preparations are indole-acetic acid (12, 46, 47), phenylacetic acid (13), ortho-hydroxy-phenylacetic acid (67), members of vitamin complexes (162), and other unidentified growth regulating factors (11, 41, 42, 48, 118, 119, 126, 129, 175). It is difficult in many cases, therefore, properly to evaluate the interpretations of the recorded results (153). In view of this fact and since extensive bibliographies of the early work have been published (14, 74, 104, 137), the present discussion is limited to studies with crystalline penicillin or to effects observed in the earlier work and subsequently substantiated with crystalline penicillin.

One current trend in research aims at identifying chemically the various substances that may be present in impure penicillins and which at proper concentrations may shorten the lag period, before the onset of logarithmic growth in cultures of bacteria. This work takes on added significance in view of the recently renewed interest in amorphous (impure) penicillin, some lots of which have been shown to contain a factor or factors that are themselves inactive but that are capable of increasing markedly the effectiveness of pure penicillin *in vitro* (57), and especially interesting, its therapeutic effectiveness *in vivo* (101, 199). Some of these enhancing factors may prove to be substances that affect the systems regulating the respiratory processes of the pathogen cells, so that the cells become more susceptible to the action of penicillin. It has been shown, for example, that small amounts of cobalt salts, which have been long known to function in oxygen transfer, when appropriately used, increase several fold the bactericidal activity of low concentrations of penicillin *in vitro* (179). Similarly the therapeutic effectiveness of penicillin *in vivo* may be doubled by appropriate use of cobalt (150). The observation that culturing bacteria in the presence of phenylacetates activates the dehydrogenases involved in the oxidation of mandelate and benzaldehyde (178) may afford a basis for elucidating the significance of phenylacetic acid and its derivatives as co-factors in "amorphous" penicillins.

It is recognized that solutions prepared from amorphous penicillin are more stable than are those prepared from crystalline penicillin (45, 132, 173). This has been ascribed to the buffering action, and consequent protective effect, of the impurities present in the amorphous product. It seems likely, however, that more than mere buffering is involved, since it has been shown that addition of small amounts of phosphate markedly stabilizes solutions of penicillin and that this effect probably cannot be accounted for entirely in terms of pH (146, 147). It appears that phosphates or other impurities that are present in amorphous penicillins may bind cations of various metals which otherwise might slowly destroy the activity of the penicillin through esterification or through opening of the thiazolidine ring. It should be pointed out that cations, such as those of heavy metals, etc., that tend to promote inactivation of penicillin during storage may actually enhance its bacteriostatic activity at the time of contact with a living bacterial cell through their ability to function as oxygen carriers (see discussion below).

#### EVIDENT EFFECTS OF PENICILLIN ON BACTERIA

*A. Morphological.*—It is well known that bacteria under the influence of bactericidal concentrations of penicillin undergo distortion and swelling and ultimate lysis (3, 10, 15, 51, 68, 69, 80, 82, 114, 117, 128, 129, 148, 172, 174, 181, 182, 194, 196). Microscopical examination of assay plates seeded with different test organisms shows that the cells not only increase in size, but tend to become concatenated. In rod forms such as *Escherichia coli* and *Bacillus subtilis* this becomes manifest by development of elongated mycelium-like structures, and in cocci by the formation of chains of cells. Thus, cells of *S. aureus* under the influence of penicillin tend to form streptococcus-like chains, concomitantly

losing their gram-positiveness (56). This is followed by swelling and abnormal enlargement of the cells. It is interesting to note that this tendency toward concatenation is also shown in streptococci which, under the influence of penicillin, tend to form longer chains than normal (80). It should be pointed out, however, that this response is not specific to penicillin, since it may be induced by sulfonamides (83, 121, 187), by norvaline (155, 189) and very markedly by bacitracin (unpublished data). At sub-bactericidal concentrations, penicillin stimulates metabolism and may act as a "growth factor" in promoting increase of cell size, although at the same time it disharmonizes the processes of cellular enlargement and cellular division.

The modification of the geometrical arrangement of the individuals in the colony may be correlated with a change in the distribution of electrostatic charges at the cell surfaces. Dorfman (49) and Dorfman and Kastorskaya (50) went so far as to attribute the action of penicillin primarily to this effect, and they proposed a method of electrokinetic assay of penicillin based on the increase of  $\zeta$  values (electrokinetic potentials) of susceptible bacteria under the influence of the antibiotic. The modification of distribution of electrostatic charges on seeded plates exposed to penicillin can be revealed by flooding the plates with suspensions of electropositive or of electronegative particles and by studying the sites of flocculation. The patterns so revealed (55) can be correlated with the patterns revealed by pH indicators and by rH indicators, and the over-all effect can be interpreted in terms of a shift of sulfhydryl ( $-\text{SH}$ ) to disulfide ( $\text{S}-\text{S}$ ), of aldehydic  $\text{COH}$  to carboxylic  $\text{COOH}$  or ketonic  $\text{CO}$ , or of enolic  $\text{COH}$  to ketonic  $\text{CO}$ .

*B. Biochemical Effects.*—Gale and Taylor (79) and Gale (77) showed that one of the earliest manifestations of the action of bacteriostatic concentrations of penicillin on *S. aureus* is a blocking of the absorption of the essential metabolite, glutamic acid. Thus their experimental evidence corroborates the earlier postulate (174) that penicillin interferes with the assimilation of essential growth factors by the organism. This effect becomes evident after very short contact of penicillin with the cell, before morphological changes are apparent. The observation that an initial biochemically evident effect of penicillin is inhibition of glutamic acid assimilation appears extremely significant, since glutamic acid is a component of glutathione, the activity of the  $-\text{SH}$  group of which is known to depend in large measure on the vicinal  $\text{NH}$  groups (85). Glutathione or similar sulfhydryl bearing proteins are known to act in the aerobic cell as reservoirs of H that are capable of promoting rehydrogenation of the dienol or aldehydic groups that are essential in aerobic respiration. In this connection it is interesting to note that Speck (177) correlated the activity of enzymatic synthesis of glutamine with proteins requiring sulfhydryl groups for full activity. Grossowicz (91) observed that *Neisseria intracellularis* grows readily in a synthetic medium containing glucose (or lactate), glutamine, thiosulfate, and mineral salts, but that it is inhibited by cystine. The dependence of this penicillin-sensitive though gram-negative organism on a reduced form of sulfur (thiosulfate) and its intolerance of a disulfide (cystine) may account for its amenable-

ness to penicillin therapy, and may provide a lead to the cytochemical mode of action of penicillin on organisms, irrespective of their reaction to Gram's stain.

Evidence from cytochemical studies indicates that penicillin exerts its bacteriostatic action by promoting dehydrogenation of  $-SH$  groups to  $S-S$  more rapidly than the organisms can restore the active sulfhydryl group (55, 56, 148, 149). This hypothesis is in full agreement with the observation that cysteine suppresses the antibacterial activity of penicillin (30-33). Cavallito and co-workers (32, 33) visualized an inactivation of penicillin in a direct stoichiometric relation and expressed some surprise at the ability of cysteine to inactivate "widely different chemical types of antibiotics", although they also recognized the possibility that penicillin action might involve interference with the normal functioning of sulfhydryl groups in bacterial metabolism. It is undoubtedly true that relatively high concentrations of cysteine may chemically inactivate penicillin over a period of hours (32, 36, 96, 133). Chow and McKee (36) showed, for example, that equimolar solutions of penicillin and cysteine react so that positive tests for  $-SH$  and  $NH_2$  in the cysteine gradually fade, the penicillin being concomitantly inactivated to penicilloic acid. However, we interpret the fact that cysteine suppresses or reverses the action of penicillin and other unrelated antibiotics (8, 9, 32), of mercurials (40, 66), and of arsenicals (58), when tested by biological assay, as evidence for an indirect action of cysteine. We propose that its effect, especially when present in low concentrations, is exerted not primarily on the antibiotic agent, but rather on the cell of the test organism. That is, *in vivo*, cysteine serves mainly as a source of  $-SH$  groups so that the organism has available a reservoir of sulfhydryl groups sufficient to fulfill its requirements, and, therefore, can tolerate concentrations of the antibiotic that would otherwise be toxic.

Mulé (134) having noted that increase in "pressure" (tension?) of hydrogen in broth cultures of *S. aureus* counteracted the bacteriostatic effectiveness of penicillin, postulated that penicillin promotes lethal dehydrogenation in the cell, which may be counteracted by providing hydrogen to activate the respiratory enzymes. He later (135, 136) more specifically inferred that penicillin acts on susceptible organisms by shifting glutathione from the reduced to the oxidized state.

There is good evidence that  $-SH$  groups are most readily demonstrated and are most reactive at the time of cellular division (27) and that concomitantly there is an active synthesis of desoxyribonucleic acid derivatives (23, 124, 159, 160, 186, 191). On the basis of this evidence, the  $-SH$  groups in organisms exposed to penicillin should be expected to suffer dehydrogenation most readily at the time of cell division. Experimental evidence fulfills this expectation. When exposed to bacteriostatic concentrations of penicillin, sensitive organisms which start to divide fail to complete the division. Thus, penicillin checks the cells in an early stage of division (24, 25, 26, 112), or perhaps even before the first division is completed (35). In this respect, penicillin differs markedly from the sulfonamides which permit several cell divisions to occur. The fact that penicillin and sulfonamides act on bacteria through different mechanisms may



account for the greater effectiveness of combinations of the two drugs than of either independently. This has been interpreted on the basis of selection and inhibition of resistant varieties (109, 109a). Although cells under the influence of penicillin are prevented from yielding two daughter cells, they do evidence excessive increase in size; they swell into diplococcus-like shells that exhibit bipolar staining with vital dyes, the color of which shifts to that indicative of a higher level of oxidation potential (148). Support for the view that there may be a distinct difference between the penicillin-cysteine relation *in vivo* and *in vitro* is suggested by the observations that inactivation of the antibiotic occurs more readily in aqueous solutions of penicillin and cysteine than in the presence of blood (97).

#### EFFECTS OF BACTERIOSTATIC AND SUB-BACTERIOSTATIC CONCENTRATIONS OF PENICILLIN

Sub-bacteriostatic concentrations of penicillin have been shown to enhance metabolic activity and growth of *S. aureus* (22, 43, 64, 65, 130, 156, 188). This accounts, in part at least, for the narrow ring of enhanced growth that immediately surrounds, or outlines, each zone of inhibition on assay plates. These rings are constant features of assay plates and can be seen on published photographs (65). It has been suggested (55, 149) that they represent regions in which the test organisms, having been subjected to sub-bacteriostatic concentrations of penicillin without subsequent exposure to "static" or "cidal" concentrations, have been stimulated to a state of intense metabolism and growth, characterized by an abnormally high rate of respiration, such as has been ascribed to the "climacteric stage" induced in cells of various tissues by traces of different chemicals. However, other factors may also contribute to the development of a ring of enhanced growth at the boundary between a region of inhibition and the circumjacent uninhibited area of the plates. Cytochemical studies have demonstrated that as cells in the area of inhibition are affected by bacteriostatic concentrations of penicillin, some of their components are liberated into the agar through which they diffuse to regions of the plate in which the concentration of penicillin fails to reach a bacteriostatic level, and it is likely that some of these substances, especially the nucleoproteins, may be absorbed by the bacterial cells outside the zones of inhibition and may serve as metabolites or growth factors. Support for such an hypothesis is provided by the experiments showing that when *S. aureus* was cultured in the presence of low concentrations of penicillin in broth, two waves of growth occurred (2, 24, 25, 200). These results may be interpreted as indicating that when cells of *S. aureus* are suspended in broth containing very low concentrations of penicillin, the most sensitive organisms which are first affected, as they undergo lysis, release into the medium substances which promote a second wave of growth among the more resistant cells. Since lysis of senescent cells normally occurs in cultures of microorganisms, penicillin may be considered as hastening the process of aging. Tulasne and Vendrely (186) have recently stressed the prominent role played by ribonuclease, actively secreted by aging bacteria, in promoting the lysis of the

extra-nuclear content of senescent bacteria, and it is apparent that this process is accelerated in the presence of appropriate concentrations of penicillin (25, 26, 64, 65, 200), making possible, under suitable conditions, post-lytic waves of growth. As early as 1945, Todd (183) had emphasized the possible role of autolytic enzymes in promoting bacteriolysis of cells exposed to penicillin. A number of references in the literature indicate that products liberated by dying microorganisms may serve as growth factors for survivors (39, 110, 122, 139, 193). The observation that in the presence of sufficiently low bacteriostatic concentrations of penicillin, proliferation of *S. aureus* in broth can continue for several hours before clearing of the suspension becomes evident (174) can be interpreted as an indication that penicillin exerts a secondary action by increasing the permeability of the most sensitive microorganisms in the colony so that some of their constituents are released into the medium where they serve to enhance the metabolic activity and to stimulate the growth of more resistant neighboring cells. Such cells would then become more susceptible to penicillin. Thus the course of events in cultures exposed to penicillin might be visualized as analogous to a "chain reaction."

#### EFFECT OF PENICILLIN ON THE RESPIRATORY SYSTEMS

From the preceding discussion, it is evident that the antibacterial effect of penicillin is more pronounced toward organisms that are growing and respiring vigorously than toward those that are in a resting stage. Culturing cells of *S. aureus* in a protein-free medium, poor in H donors, rapidly brings them to a resting stage, whereupon the respiratory activity falls progressively (Schuler, 167). The rate of fall is not appreciably affected by addition of penicillin (167, fig. 14). Conversely, in nutrient broth, oxygen uptake which is low during the first half hour, increases logarithmically with time, and reaches a maximum about the third hour; this is the time when the density of population of living cells is greatest. Addition of penicillin to the cultures just before or during the logarithmic phase shortens the duration of that phase of respiration and growth, the curtailment being directly related to the concentration of penicillin. Schuler (168, 169) obtained similar curves using streptomycin. Hirsch and Dosdogru (98) duplicated the work of Schuler (167, 170), and it is interesting to note that the corresponding curves are virtually superimposable. In both cases, enhancement of respiration is apparent in organisms exposed to very low concentrations of penicillin, thus providing experimental support for our hypothesis (55, 56, 148, 149) based on interpretation of the color reactions obtained on assay plates treated with suitable dyes and indicators. Schuler further showed that the curve relating evolution of  $\text{CO}_2$  to time is also affected by addition of penicillin. The curves for both  $\text{O}_2$  consumption and  $\text{CO}_2$  evolution decline after the period of maximum activity at about three hours has been passed. The bacteriostatic effect of penicillin is less marked in broth saturated with  $\text{CO}_2$  than in aerobic controls, as shown by the fact that the curve for  $\text{CO}_2$  evolution falls more abruptly in the aerobic cultures than in those saturated with  $\text{CO}_2$  (167, fig. 10). This is completely in accord with the more recent conclusion of Mulé (134) that the

bacteriostatic effectiveness of penicillin is a direct function of  $O_2$  tension in the broth. Schuler (168) later showed that the responses of *E. coli* and of *S. aureus* to penicillin are similar, although the concentrations necessary to induce the typical effects are considerably higher for *E. coli*. Independently, from cytochemical studies, we reached a similar conclusion, i.e., that penicillin affects gram-positive and gram-negative organisms through the same mechanism (149), the principal difference being in the concentration of antibiotic required. From studies with *S. aureus* in the Warburg apparatus (92) it appears that in this organism, as is well known for aerobic cells in general, active  $O_2$  uptake is dependent upon the activity of a cytochrome reducing dehydrogenase-cytochrome-cytochrome-oxidase system in a chain of reactions as portrayed by Grumbach (92). The strong positive reactions given by actively growing *S. aureus* with solutions of tannin, ferricyanide, hematoxylin, and other reagents that form colored iron complexes may be interpreted as further support for the prevalence of iron-protein enzymes in these organisms.

The observation (54, 144, 167, 168) that penicillin inhibits  $O_2$  uptake most effectively in actively growing bacterial cells suggests that penicillin may block

TABLE 3

*Diameters of zones of inhibition surrounding penicylinders\* containing different concentrations of KCN*

ORGANISM	PER CENT CONCENTRATION OF KCN		
	0.001	0.01	0.1
<i>S. aureus</i> .....	14.5 mm	20.4 mm	24.2 mm
<i>E. coli</i> .....	10.5 mm	12.3 mm	19.0 mm

\* Outside diameter 8 mm.

the cytochrome-cytochrome-oxidase system. Since this is the portion of the respiratory mechanism that is known to be cyanide sensitive, it seemed of interest to compare the reactions of the penicillin-sensitive *S. aureus* and the penicillin-resistant *E. coli* to cyanide, which has been reported to stimulate oxidation of internal material while inhibiting oxidation of the external substrate (63, 84). This was done by means of a modification of the cylinder-plate technique for assaying penicillin. Seeded plates were incubated for three hours; cylinders were placed thereon and were filled with appropriate solutions of KCN. Then the plates were reincubated for five hours. Following incubation, treatment with Schiff's reagent, the ferricyanide- $FeSO_4$  reagent for Prussian Blue, or other suitable reagents, revealed a contrast between the inhibition zone which remained uncolored and the background which gave a positive color reaction. As shown in table 3, application of a given concentration of KCN resulted in considerably larger zones on plates seeded with *S. aureus* than on those seeded with *E. coli*. The curves relating log diameter of inhibition zone to log dose are homologous, however, just as is true for penicillin.

Similar results were found with  $NaN_3$  toward which, however, the organisms

appeared to be less sensitive. Application of suitable staining techniques previously described (55, 56) showed that the bacteriostatic action of diffusing KCN, like that of penicillin, eventually results in dehydrogenation of sulfhydryl groups of the test organisms. The threshold concentration for antibacterial action of KCN and of  $\text{NaN}_3$ , as was true for penicillin, appeared to be much lower and more clearly defined for *S. aureus* than for *E. coli*. However, once the threshold concentration has been exceeded, the aerobic respiratory systems become unbalanced, —SH groups are used up faster than they can be restored, and the oxidation-reduction potential rises above a level compatible with life. Thus, agents favoring dehydrogenation of —SH groups should be expected to potentiate the action of penicillin. This may afford an explanation for the recorded observation that dyes in the oxidized state (capable of acting as hydrogen acceptors) enhance the action of penicillin (185). The observation of Quastel and Yates (152) that certain basic dyes of the triphenylmethane series inhibit  $\text{O}_2$  uptake in *E. coli* seems pertinent in this connection. Although the full significance of the fact is not apparent at present, it seems worthy of comment that the potentiating action of dyes on penicillin effectiveness is most pronounced against gram-negatives, and that the dyes which are most effective are those which have an affinity for the gram-positive complex. Just as dehydrogenation of —SH groups more rapidly than they are restituted should be expected to enhance the action of penicillin, so binding or otherwise removing —SH groups from active participation in the reversible metabolic processes of the cell should be expected to make penicillin inhibitory at lower concentrations. Evidence that this may occur is found in the fact that bismuth (115, 116) and cobalt (150, 179) in concentrations which are not inhibitory in themselves, do enhance the effectiveness of penicillin action. Likewise, agents that might tend to "unfold" —SH bearing proteins causing the thiol groups to become exposed and more accessible to dehydrogenation should be expected to potentiate the action of penicillin. This may afford an explanation for the synergistic action of detergents such as cetylpyridinium with penicillin (185).

Numerous enzyme systems involved in carbohydrate, nitrogen, and fat metabolism contain essential thiol groups. Simpler metabolites such as cysteine and glutathione (gamma glutamyl-cysteinyl-glycine) also owe many of their biochemical properties to the presence of a thiol group. In fact, it has been suggested that the primary function of glutathione, which is itself a coenzyme for the glyoxalase enzyme system, is the continuous reactivation of —SH cellular enzymes. Many microorganisms require an external source of potential organic thiol groups, and glutathione has been shown to be a growth factor for variant strains of gonococcus (157, 158). The structure of the penicillin molecule can be written in such a way as to show a striking resemblance to glutathione, and it may be suggested that penicillin may compete with glutathione in processes involving H transfer.

The apparent oxidation-reduction potential of reduced glutathione is low enough in the  $E'_0$  scale for the threshold mechanism, postulated for the bacteriostatic mechanism of penicillin toward aerobes, to be operative toward anaerobes

also but at a lower level so that the flavin system may be expected to be involved.

#### EFFECTS OF PENICILLIN INCIDENT TO ALTERED RESPIRATION

Any agent that impairs  $O_2$  uptake correspondingly impairs the ability of the aerobic cell to absorb and to retain ions or molecules. Thus loss of ability to accumulate vital dyes or loss of ability to absorb silver nitrate (anargyrophily) may be used as a measure of inhibition of respiration, and specifically, can be used as a measure of the bacteriostatic effect of penicillin on suitable test organisms. It may be further used to explore the inhibition of a given enzyme, and to correlate such an enzyme with the production of the energy required for the absorption of ions and molecules from the environment, and their accumulation against the concentration gradient. The inhibition of the energy-providing respiratory system may be so great as not only to prevent absorption and retention of materials from the external environment, but to prevent retention by the cells of their normal constituents (56, 148). This is easily demonstrated by treating the test organisms with a solution of silver nitrate or silver chloride. Actively growing organisms manifest argyrophily, that is to say they absorb silver salts which are deposited as vacuolar precipitates of metallic silver. As test organisms undergo the swelling preliminary to lysis, they allow some of the reducing cellular material to leak out of the cells, and to induce an extracellular deposition of silver; therefore, a kind of silver plating occurs around the cells in the pre-lytic state and in their immediate vicinity. This is the basis of a three-hour cylinder-plate assay for penicillin (89). Ultimately, when the oxidation potential has been shifted upwards sufficiently, the cells lose their ability to promote silver deposition; in other words, they reach the stage of anargyrophily (161).

The reducing ability of actively growing *S. aureus* is exhibited similarly in its capacity to reduce nitrate to nitrite: that capacity is inhibited by penicillin in proportion to its concentration, a fact which has been used for quantitative estimation of penicillin (88). Similarly, anaerobes in the presence of penicillin lose their ability to obtain molecular oxygen from appropriate donors. Therefore, proper use of a suitable dye, such as Janus green, as an oxygen donor makes possible quantitative assay of penicillin against anaerobes (151).

When assay plates seeded with aerobic test organisms are flooded with a solution of oxidized Janus green the dye remains oxidized (green) in the inhibition zone, but is reduced almost immediately by the actively growing organisms outside the zones to the pink quinonoid which is intermediate between the fully oxidized (green) and completely reduced (leuco) forms of the dye. Conversely when plates are flooded with a solution of the leuco dye (completely reduced form) a pink color immediately develops in the actively growing colonies outside the zones of inhibition. Thus it appears that in aerobes growing actively on a nearly neutral medium, the potential is above that of the reduced dye but is below that of the oxidized form.

COMPARATIVE EFFECTS OF PENICILLIN UNDER AEROBIC (PETRI PLATES)  
AND SEMI-ANAEROBIC (BROTH) CONDITIONS

It may be assumed that on penicillin assay plates there is a uniform oxygen tension and that there is a gradient of penicillin concentration which decreases from the cylinders outward. Conversely, it may be assumed that in broth cultures, there is a uniform penicillin concentration in the medium but that there is a gradient of oxygen tension which decreases from the surface downward. An interesting comparison and demonstration of a critical oxidation-reduction potential threshold may be made by applying appropriate redox indicators to cultures on standard assay plates and in serial dilutions.

When assay plates seeded with *S. aureus* and incubated for 16 hours, as in the standard cylinder-plate assay, are flooded with appropriate oxidation-reduction indicators each zone of inhibition is promptly outlined by a ring that is the site of enhanced activity of the cells and that indicates the position of an rH threshold above which the cells are unable to grow (inside of zone) and below which they grow normally (uninhibited background). Likewise, if equal volumes of broth containing different amounts of penicillin are inoculated with equal numbers of organisms of the same age and are incubated until marked bacteriostasis is evident in the tube with the highest concentration of penicillin, addition of a suitable indicator reveals that an rH threshold has been established in each tube and that the depth of this threshold below the surface increases as the concentration of penicillin increases. In fact the depth of the "oxidized" layer is a linear function of the log of the concentration of penicillin in the broth. The more active the dehydrogenases in a given tube, the closer to the surface will a given indicator be reduced to the leuco-compound. The concentration of antibiotic in the broth can, therefore, be expressed in terms of the depth of the layer of the unreduced dye in the upper layer of the suspension.

A comparison of results obtained from *S. aureus* 16-hour assay plates and serial dilution assays treated with a mixture of dimethyl-paraphenylenediamine hydrochloride and thymol is diagrammed in figure 1. Similar results were obtained using Janus green or Indigosol green IB (obtainable from Durand and Hugenin, Basel, Switzerland). The upper row represents the inhibition zones surrounding cylinders containing geometrically increasing concentrations of penicillin on standard 16-hour assay plates. Correspondingly, the lower row represents the depth of the layer of "oxidized" dye in the several tubes. The numerals indicate the concentration of penicillin, in units per ml, in the different cylinders or tubes, which have been spaced on a logarithmic scale in the diagram. The approximately linear relation between response (diameter of zone or depth of "oxidized" layer) and log concentration of penicillin is indicated by the tangent to the inhibition zones on plates, and by the line through the lower edges of the layers of "oxidized" dye in the tube experiments.

Any agent which may serve in oxygen transfer accelerates dehydrogenation in the cells and favors the loss of reducing capacity of the cells. Thus it tends to increase the action of a given concentration of penicillin. For example, co-

baltous salts increase two to four or more times the response *in vitro* to penicillin, and its effectiveness *in vivo* (150, 179).

#### INFLUENCE OF PENICILLIN ON METABOLISM OF GLUTAMIC ACID

It is interesting to note that Gale (77, 78) and Gale and Taylor (79) observed that failure of cells of *S. aureus* to assimilate glutamic acid was among the first physiologically manifested effects of penicillin action. The significance of this is emphasized by the earlier observation that penicillin-insensitive gram-negative organisms such as *E. coli* are even more insensitive to penicillin in the presence of

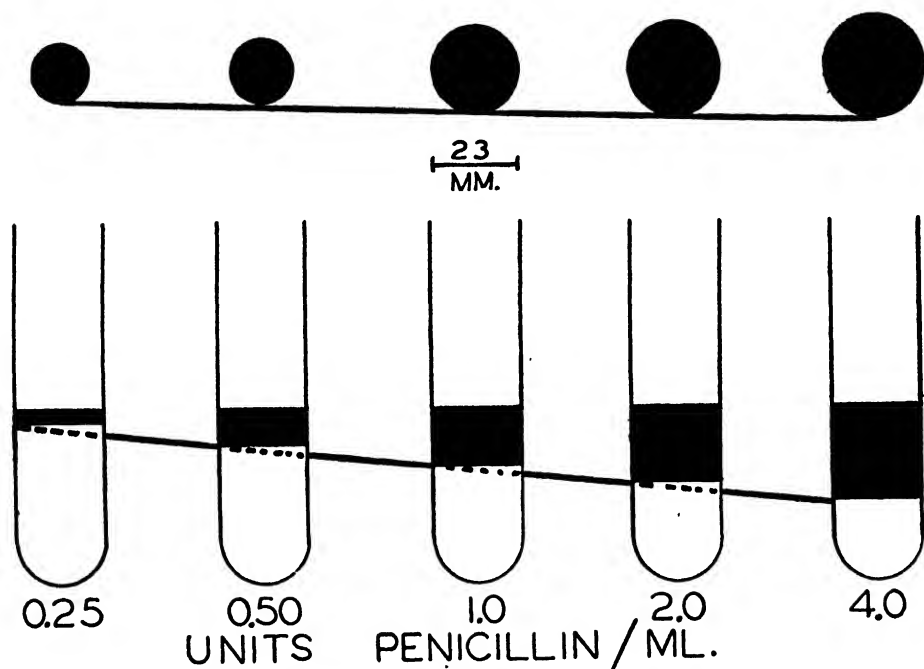


FIG. 1. DIAGRAMMATIC REPRESENTATION OF INHIBITION ZONES ON PENICILLIN ASSAY PLATES AND OF "OXIDIZED" LAYERS IN SERIAL DILUTION ASSAYS IN BROTH, FOLLOWING ADDITION OF SUITABLE DYE  
(See text for explanation)

glutamic acid (171). The depressing effect of glutamic acid can be blocked or counteracted by the antimetabolite, methionine; in fact, methionine markedly sensitizes gram-negatives toward penicillin (171). At present it is difficult to account for this effect. It should be pointed out, however, that methionine is known to be mostly effective in transmethylation, and the mechanisms of its synergistic effect with penicillin should be studied in this light. The importance of the position of the methyl groups is demonstrated by the fact that *dl*-thiazolidine-4-carboxylic acid in a 1:500 solution is bacteriostatic toward *S. aureus* whereas the 2-dimethyl derivative is inactive even at a concentration of 1:100 (138). Conversely, valine which may be considered to be an analog of the thia-

zolidine ring in the penicillin molecule has been shown in our laboratory to exhibit pronounced inhibition of *S. aureus* on assay plates in concentrations of 1:100,000. Most important among the metabolic processes are transamination, transmethylation, decarboxylation, phosphorylation, and acetylation. Transmethylation has been discussed above in relation to the effect of methionine in association with penicillin. Interference with transamination and decarboxylation might be considered specifically in connection with the inhibition of glutamine metabolism, through interference with the aldehyde or amine pyridoxine derivatives.

Transamination and decarboxylation depend on the phosphorylation of pyridoxal into the active coenzyme (108, 176). It has been shown recently that the inactive apoenzyme of a transaminase system, on addition of pyridoxal phosphate, may reconstitute an active glutaminase or an active tryptophanase (44). Addition of diphosphopyridine nucleotide to the apoenzyme is equally effective. The discussion thus far has been focussed on the interference of penicillin with H transfer. However, phosphorylation, resulting from the proper transfer of  $\text{PO}_4$  is as fundamentally important as hydrogenation. It may be suggested that just as supplying the proper H donor may enable microorganisms to survive in the presence of otherwise inhibitory or "cidal" concentrations of penicillin, so supplying suitable phosphorylated compounds (such as nucleic acid) may be similarly effective. This may explain why nucleic acid antagonizes the bacteriostatic action of penicillin (143).

It may be postulated that the different reactions of gram-positive organisms to penicillin as compared with gram-negatives may depend on the different nature of the nucleotides that are present. Examples of differences in the reactivity of nucleotides from different sources with penicillin and other bacteriostatic agents have been reported (14, 90).

Future investigation may show acylation to be also of fundamental importance. Cytochemical evidence from assay plates treated with phenolphthalein phosphate in our laboratory has shown that test organisms outside of the zones of inhibition contain an active alkaline phosphatase, while no trace of phosphatase activity can be demonstrated within the zones of inhibition. Recent studies have emphasized the importance of phosphatases in microorganisms, and in view of the general significance of the energy-rich phosphate bonds in all physiology (120), it appears that further studies may clarify the interrelationship of antibiotics such as penicillin with phosphatases as well as with transaminases. Since penicillin affects cholinesterase in experimental animals (76), it may be suspected of interfering with acetyl-phosphatase activity of microorganisms.

#### CONCLUSION

The following facts appear to be clearly established by reports in the literature or by our own research:

1. Microorganisms which are metabolizing actively, i.e., those actively absorbing  $\text{O}_2$  and evolving  $\text{CO}_2$ , are most sensitive to the action of penicillin.
2. At a given concentration of penicillin, organisms become more susceptible



to its action as the concentration of  $O_2$  available to the test organisms is greater, or as there is also present some system capable of activating the transfer of  $O_2$ .

3. Appropriate sub-bacteriostatic concentrations of penicillin markedly increase the reducing capacity of microorganisms, and this may be interpreted as indicating increased demand for oxygen.

4. At bacteriostatic concentrations, however, penicillin inhibits  $O_2$  uptake and  $CO_2$  release.

5. Thus there results an irreversible dehydrogenation of aldehyde groups and of  $-SH$  groups, and consequently the oxidation potential is shifted to a level above that compatible with the proper functioning of the respiratory mechanism. The observations of Prévot (151) suggest that the same threshold mechanism operates for anaerobes as well as for aerobes but at a lower  $rH$  value.

Penicillin apparently tends to enhance dehydrogenation in the so-called penicillin-fast organisms, or even in cells of tissues. Ability to survive or even thrive in the presence of penicillin may be correlated with cytochemical ability to restore  $-SH$  groups fast enough for the respiratory systems to remain poised within the limits of reversible dehydrogenation and rehydrogenation. An organism may be assumed to be penicillin-sensitive when penicillin stimulates dehydrogenation of its functional sulfhydryl groups faster than they can be restored. Decreasing the rate of  $H$  transfer or making extraneous  $-SH$  groups available nullifies the effect of penicillin. Increasing the rate at which  $H_2$  becomes accepted by oxygen transporters or donors potentiates the effectiveness of penicillin. Factors which "expose"  $-SH$  groups formerly protected in protein molecules, such as detergents, bacteriophage, etc., increase the sensitiveness of the organisms to penicillin.

The sensitive cell is induced into the lethal dehydrogenation of its essential constituents, while releasing into the medium growth-promoting substances which induce nearby cells into a stage of metabolism in which they are penicillin-sensitive. In that sense paraphrasing and adding to the *British Medical Journal* (October 6, 1945, p. 465) it is difficult to absolve the cell from a charge of collaboration not only in its own death but in that of its relatives.

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# SURFACE ACTIVE AGENTS AND THEIR APPLICATION IN BACTERIOLOGY

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Surface active agents have a wide range of utility as manifested by their applications in detergency, solubilization, emulsification, capillary penetration, wetting, and spreading. Because of their growing industrial utilization these compounds have become commercially available on an increasing scale during the past twenty years with consequent opportunities for application to systems of biological interest. In interaction with such systems, these compounds have exhibited marked effectiveness in low concentrations, with phenomena such as precipitation, complex formation and denaturation of proteins, cytolysis of cells, destruction of microorganisms, and inactivation of viruses as examples.

It is the purpose of this review to present the chemical structure and physical properties of surface active agents in relation to their biological activity. The effects of surface active compounds on proteins are discussed briefly. A more comprehensive discussion of the interactions of proteins and surface active agents may be found in a current review by Putnam (185).

Before starting on the main thread of development of this review it may be well to define a few of the terms which will find constant usage. This is an especial necessity because numerous terms have been utilized to characterize certain industrially important activities of these compounds, and, through widespread usage, have taken on meanings of a more generic nature than was originally intended.

Surface active agents may be defined as substances which alter the energy relationships at interfaces. Among the manifestations of these altered energy relationships is the lowering of surface or interfacial tension. More specifically,

the following types of important industrial usages of these compounds are recognized:

- (a) Wetting Agents. Promoters of spreading of liquids on surfaces or of penetration of liquids into materials.
- (b) Detergents. As in the cleansing of dirt from textiles.
- (c) Emulsifying Agents. Aids in the dispersion of one phase within another, ordinarily immiscible, phase.

All the above types of compounds are surface active, but the utility of any compound as a wetting agent, detergent, or emulsifying agent is an expression of an aggregate of properties, including specific chemical configuration, and is inadequately expressed by any one simple measurement such as surface tension lowering. Thus, two compounds may display an equal ability to lower surface tension, with one being an efficient wetting agent, whereas the other may be quite deficient in this respect (for an excellent example see Wilkes and Wickert (242)).

For these reasons the term surface active agents has been chosen as the most general appellation of those compounds. Such terms as wetting agents, detergents, and emulsifying agents should be reserved for denoting specific functions of a given compound. It is well to keep in mind, however, that this has not been scrupulously observed in the literature and, especially in work of a biological background, the term detergent is often used synonymously with surface active agent.

Compounds displaying surface activity are characterized by an appropriate structural balance between one or more water-attracting groups and one or more water-repellent groups. Various synonyms have been used for the water-attracting groups. They have been known as hydrophilic or polar groups. Similarly, the water-repellent groups have been known as hydrophobic, non-polar, or hydrocarbon groups. For purposes of this review the terms *hydrophilic* and *hydrophobic* will be used.

#### CHEMICAL STRUCTURE AND PHYSICAL PROPERTIES OF SURFACE ACTIVE AGENTS

##### *Classification of Surface Active Agents*

The electrical charge on the hydrophilic portion of a surface active agent may serve as a convenient basis of classification of these compounds. Dependent upon the nature of this charge, or the absence of ionization, surface active agents have been classified as: Anionic, Cationic, Non-Ionic, or Amphoteric.

An *anionic* surface active agent is characterized by a structural balance between a hydrophobic residue (e.g., paraffinic chain, alkyl substituted benzene or naphthalene ring) and a negatively charged hydrophilic group (e.g., carboxyl, sulfate, sulfonate, or phosphate). In *cationic* surface active agents the same hydrophobic residues may be balanced with a *positively* charged hydrophilic group (e.g., quaternary ammonium, sulfonium, arsonium, phosphonium or iodonium). *Non-Ionic* surface active agents possess no ionized groups. The hydrophobic portion of a non-ionic surface active agent is balanced by such non-ionized hydrophilic groups as polymerized ethylene oxide or polyhydric alcohols. *Amphoteric* surface active agents are compounds of mixed cationic-

anionic structure. This latter type of compound is of no practical importance at the present time.

A brief outline has been compiled of the type structures of various commercially important surface active agents (adapted from Price (184)). It should be emphasized that this outline (see figures 1-6) is illustrative only and does not attempt to list all the types of chemical structures which might display surface activity. Paraffinic carbon chains are represented by rectangular strips in the figures.

A few commercially available examples of each type compound are listed below.

*Paraffin Chain Salt Types* (see fig. 1 and table 1)

*Soaps:* Sodium oleate

*Alkyl Sulfonates:* Fatty acid sulfonate ('arctic syntax A')

*Alcohol Sulfates:* Lauryl sulfate ('duponol WA')

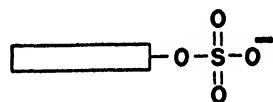
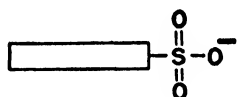


FIG. 1

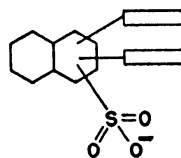
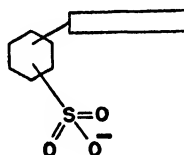


FIG. 2

FIG. 1. ANIONIC SURFACE ACTIVE AGENTS. Paraffin chain salt types. Top: soap; center: alkyl sulfonate; bottom: alcohol sulfate.

FIG. 2. ANIONIC SURFACE ACTIVE AGENTS. Alkyl aryl sulfonates. Top: alkyl benzene sulfonate; bottom: alkyl naphthalene sulfonate.

*Alkyl Aryl Sulfonates* (see fig. 2 and table 1)

*Alkyl Benzene Sulfonates:* Dodecyl-benzene sulfonate ('santomerse 3')

*Alkyl Naphthalene Sulfonates:* ('alkanol B')

*Paraffin Chain Salts With Complex Hydrophilic Groups* (see fig. 3 and table 1)

Amide of oleic acid and methyl taurine ('igepon T')

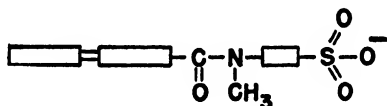


FIG. 3. ANIONIC SURFACE ACTIVE AGENTS

Paraffin chain salts with complex hydrophilic groups: amide of oleic acid and methyl taurine.

*Compounds With Hydrophilic Groups Near Middle of Hydrocarbon Chain* (see fig. 4 and table 1)

## Dioctyl-sulfosuccinate ('aerosol OT')

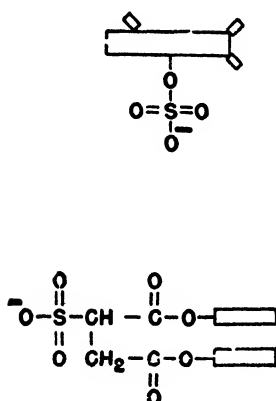


FIG. 4

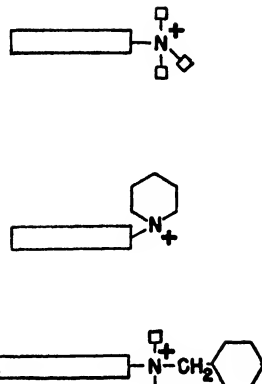


FIG. 5

FIG. 4. ANIONIC SURFACE ACTIVE AGENTS. Compounds with hydrophilic groups near middle of carbon chain. Top: sulfate of 3,9-diethyl-tridecanol-6; bottom: dialkyl sulfosuccinate.

FIG. 5. CATIONIC SURFACE ACTIVE AGENTS. Top: alkyl derivatives of aliphatic amines; center: alkyl derivatives of aromatic amines; bottom: alkyl-aryl derivatives of aliphatic amines.

*Cationic Surface Active Agents* (see fig. 5 and table 2)

*Alkyl Derivatives of Aliphatic Amines:* Cetyl-trimethylammonium (CTAB)

*Alkyl Derivatives of Aromatic Amines:* Cetyl-pyridinium ('ceepryn')

*Alky-Aryl Derivatives of Aliphatic Amines:* Cetyl-dimethylbenzylammonium

*Non-Ionic Surface Active Agents* (see fig. 6 and table 3)

*Partial Esters of Polyhydric Alcohols with Fatty Acids:* 'Polyethylene glycol 400 monolaurate'

*Esters of Polyhydric Anhydrides and Fatty Acids:* Sorbitan mono-oleate ('span 80')

*Polyether Alcohols:* Polymerized ethylene oxide condensate ('igepal CA')

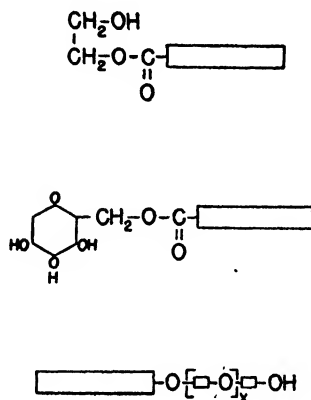


FIG. 6. NON-IONIC SURFACE ACTIVE AGENTS

Top: partial esters of polyhydric alcohols and fatty acids; center: esters of polyhydric anhydrides and fatty acids; bottom: polyether alcohols.

*Selected List of Commercial Surface Active Agents and Their Properties*

No attempt has been made to present a complete listing of commercially available surface active agents. Indeed, with the rapid appearance of new compounds, any attempt would soon be outdated. Instead, a list of 10 anionic, 10 cationic, and 6 non-ionic surface active agents, selected to illustrate a variety of structures, has been drawn up.<sup>1</sup>

TABLE 1  
*Characteristics of selected anionic surface active agents*

TRADE NAME	MANUFACTURER	CHEMICAL DESCRIPTION	ACTIVE AGENT	SURFACE TENSION (DYNES/CM AT 25 C) AT THE FOLLOWING CONCENTRATIONS (IN PER CENT)					
				1.0	0.1	0.01	0.001	0.0001	0.00001
Aerosol OT	American Cyanamid	Diocetyl sodium sulfosuccinate	100	26.3	29.9	43.0	56.8	63.0	72.1
Alkanol B	du Pont	Alkyl-naphthalene sulfonate	?	31.8	44.1	61.3	70.6	71.5	71.6
Arctic Syntex A	Colgate	Fatty acid sulfonate	85	28.2	28.9	33.8	56.5	70.3	71.2
Aresklene 400	Monsanto	Dibutyl-phenylphenol sodium disulfonate	100	29.2	34.1	45.8	61.6	70.9	71.7
Duponol WA	du Pont	Sodium lauryl sulfate	?	28.7	32.0	46.2	64.3	71.5	71.8
Igepon T	General Dye-stuff	Sodium salt of amide of oleic acid and methyl taurine	?	27.7	29.6	35.7	55.4	71.0	71.5
Santomerse 3	Monsanto	Dodecyl-benzene sulfonate	100	31.0	33.4	41.8	60.0	70.0	70.9
Sodium oleate	Eimer and Amend	Same as trade name	100	25.0	25.0	30.0	48.0	68.5	71.5
Tergitol 7	Carbide and Carbon	Sodium sulfate derivative of 3,9-diethyltridecanol-6	25	25.8	28.5	44.2	54.7	66.0	71.1
Victawet 58 B	Victor	Phosphorated capryl alcohol	70	22.8	24.9	36.0	58.9	70.5	72.0

Note: Surface tension of water at 25 C is 72.0 dynes/cm.

To make the listing more useful certain basic data have been obtained for each of the compounds (65). Trade names, manufacturers, chemical descriptions, percentage of active material,<sup>2</sup> and surface tension measurements at six concentration levels are listed for anionic compounds in table 1, for cationic compounds in table 2, and for non-ionic compounds in table 3.<sup>3</sup> It is interesting

<sup>1</sup> For an extensive listing of trade names, chemical descriptions, and manufacturers see (141).

<sup>2</sup> In those cases where it was impossible to ascertain the percentage of active material (indicated by ? in the tables), it was assumed that the commercial samples were 100% active.

<sup>3</sup> Surface tension was determined with the du Nouy tensiometer calibrated against water and benzene as standards.



to note that, despite marked differences in chemical configuration, similar ranges of surface activity may be obtained in all three classes of surface active agents.

TABLE 2  
*Characteristics of selected cationic surface active agents*

TRADE NAME	MANUFACTURER	CHEMICAL DESCRIPTION	ACTIVE MATERIAL	SURFACE TENSION (DYNES/CM AT 25 C) AT THE FOLLOWING CONCENTRATIONS (IN PER CENT)						
				1.0	0.1	0.01	0.001	0.0001	0.00001	
Ceepryn	Merrell	Cetyl-pyridinium chloride	%	?	38.5	40.7	51.2	67.0	69.8	71.3
Cetyl dimethylamine oxide	Onyx	Same as trade name	20	27.5	28.5	30.1	40.7	70.3	72.0	
Cetyl dimethylbenzyl ammonium chloride	Onyx	Same as trade name	25	30.9	31.1	34.4	56.2	69.6	70.7	
CTAB	J. T. Baker	Cetyl-trimethyl ammonium bromide	%	?	33.9	35.3	46.5	65.9	70.5	71.3
Ethyl cetab	Rhodes	Cetyl-dimethyl-ethyl ammonium bromide	100	32.5	33.3	36.0	54.7	70.2	71.5	
Ethyl decab	Rhodes	9-octadecenyl-dimethylethyl ammonium bromide	100	32.1	32.7	41.4	66.5	70.2	71.3	
LPC	Hooker	Lauryl-pyridinium chloride	26	40.8	38.0	55.7	68.7	71.3	71.6	
Octab	Rhodes	Octadecyl-dimethylbenzyl ammonium chloride	100	32.9	34.5	37.0	59.9	68.5	71.2	
Phemerol	Parke-Davis	p-tertiaryoctyl-phenoxyethoxyethyl-dimethylbenzyl ammonium chloride	100	36.4	36.2	52.0	62.5	69.0	72.0	
Roccal	Winthrop	Alkyl-dimethylbenzyl ammonium chloride	20	31.6	32.2	40.5	61.9	69.5	71.5	

*Physical Properties of Solutions of Surface Active Agents*

Studies of the physical properties of solutions of surface active agents have revealed a number of anomalies, with the experimental facts being largely unchallenged but the underlying theory still nebulous and, to a large extent, highly controversial. The behavior of extremely dilute solutions of ionic surface active agents has been found to approximate that of an ordinary strong electrolyte (KCl) but, as the concentration increases, marked divergencies in such

physical properties as equivalent conductivity, ionic transference number, osmotic coefficient, and surface tension become apparent (136, 195).

As an illustration, the equivalent conductivity of a typical homologous series of cationic surface active agents, amine hydrochlorides, may be presented (196). Figure 7 represents the equivalent conductivities as a function of the square root of the concentration of an homologous series of amine hydrochlorides over the range  $C_8$  to  $C_{18}$ . A portion of the same data has been plotted on an enlarged scale and the theoretical Onsager slope for each curve included in figure 8. Three ranges are demonstrated by these results. In the first range the equivalent conductivity falls as a linear function of the square root of the concentration

TABLE 3  
*Characteristics of selected non-ionic surface active agents*

TRADE NAME	MANUFACTURER	CHEMICAL DESCRIPTION	ACTIVE MATERIAL	SURFACE TENSION (DYNES/CM AT 25 C) AT THE FOLLOWING CONCENTRATIONS (IN PER CENT)					
				1.0	0.1	0.01	0.001	0.0001	0.00001
Carbowax 1500 diolate	Glyco Products	Oleic acid ester of a polymerized polyethylene glycol	?	32.0	33.7	36.3	37.4	63.0	71.8
Igepal CA	General Dye-stuff	Polymerized ethylene oxide condensation	?	30.3	30.2	40.7	59.7	71.0	72.0
Polyethylene glycol 400 mono-laurate	Glyco Products	Lauric acid ester of a polymerized polyethylene glycol	?	31.8	34.1	36.5	53.7	68.5	71.2
Span 80	Atlas Powder	Sorbitan mono-oleate	100	29.0	29.5	30.7	57.5	71.4	71.7
Tween 80	Atlas Powder	Sorbitan mono-oleate polyoxyalkylene derivative	100	40.4	40.2	44.6	51.5	70.3	72.0
Triton A-20	Rohm and Haas	Polyether alcohol	25	33.7	36.2	43.5	59.3	71.3	71.4

similar to the behavior observed in strong electrolytes. At a concentration which has been designated the "critical concentration" and which is dependent upon the length of the paraffin chain, the temperature, and the ionic environment, a sharp break from the behavior expected of strong electrolytes is observed. In the example illustrated, the "critical concentrations" are 0.04 M for the  $C_{10}$ , 0.013 M for the  $C_{12}$ , 0.004 M for the  $C_{14}$ , 0.0008 M for the  $C_{16}$ , and 0.0003 M for the  $C_{18}$ . A rapid fall in equivalent conductivity with increasing concentration characterizes this second range. In the third range the equivalent conductivity remains constant or rises. Results similar to these in character have been obtained in studies on anionic surface active agents (249).

The basis of explanation for these marked deviations from expected behavior has been the formation of aggregates, designated as micelles. McBain postu-

lates the existence of spherical ionic micelles and much more poorly conducting lamellar micelles. Both types of micelles are considered coexistent in solution, the relative amounts of each dependent upon the nature of the solution and the temperature (136). Hartley (74), on the other hand, believes that these effects can be explained by predicating only one type of colloidal particle, a spherical

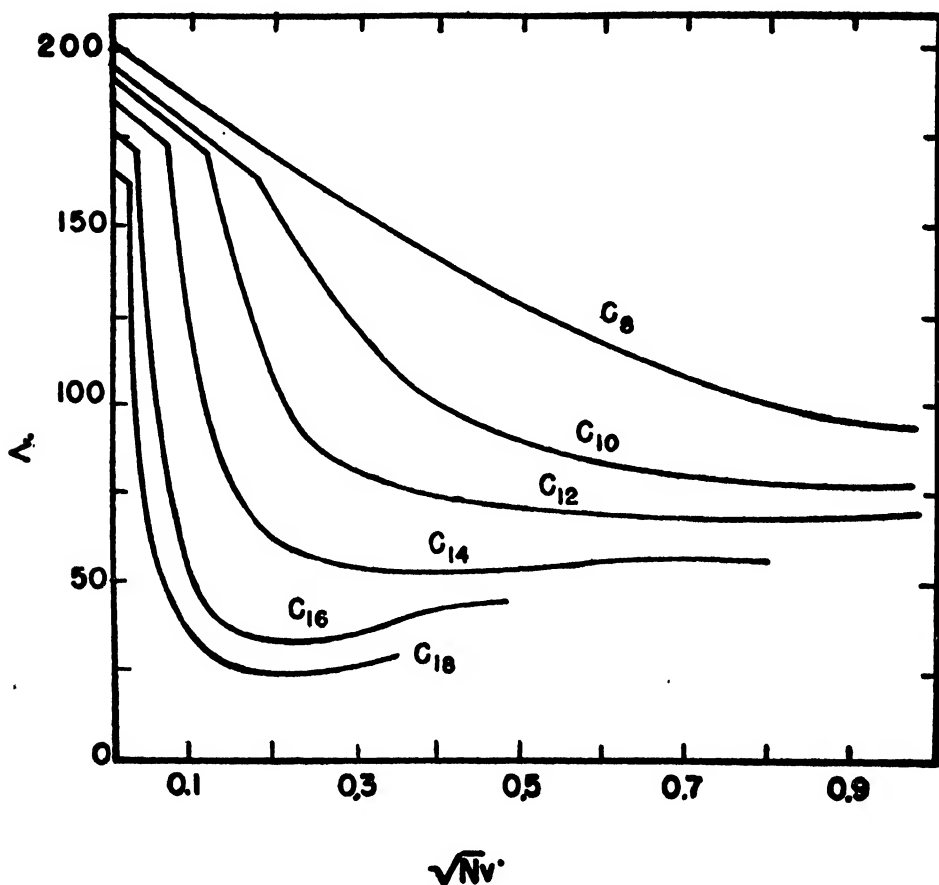


FIG. 7. EQUIVALENT CONDUCTANCES AT 60°C OF AN HOMOLOGOUS SERIES OF ALKYL AMINE SALT SOLUTIONS ( $C_nH_{2n+1}NH_2 \cdot HCl$ )

The number of carbon atoms in the alkyl portion of the molecule is indicated on each curve. Adapted from (196).

ionic micelle formed by the association of large ions. To this spherical micelle are attached a number of oppositely charged ions, so-called "gegen-ions". Anomalous conductivity effects are thought to be due to changes in the degree of ionization of the "gegen-ions". Although recent x-ray studies indicate the presence of both spherical and lamellar particles in solutions of colloidal electrolytes, adherents of both of the above views on micellar structure may still be found.

While this discussion has concerned itself with the ionized surface active agents,

it can be demonstrated by cryoscopic measurements that their non-ionic counterparts are also characterized by micelle formation, a critical concentration, and expansion of micellar structure with dilution (67).

### *Structure of Surface Active Agents and its Relation to Function*

#### *a. Anionic Surface Active Agents*

**Chain Length.** For the anionic surface active agents with long paraffinic chains in the hydrophobic portion of their molecules, it has been found that, other things being equal, the length of the carbon chain has an important influence upon the degree of surface activity of the compound. Surface and interfacial tension measurements have demonstrated increasing surface activity as chain length is increased, over the range  $C_8$  to  $C_{16}$ , with a maximum being reached

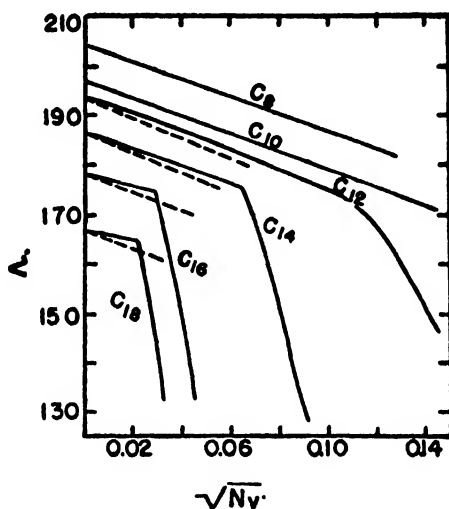


FIG. 8. ENLARGED PLOT OF PORTION OF DATA IN FIGURE 7

The broken lines indicate the Onsager values for each salt: Adapted from (196).

at  $C_{14}$  or  $C_{16}$ . Above  $C_{16}$  surface activity decreases. This importance of carbon chain length in determining surface activity has been demonstrated with soaps (213), alcohols (34, 39, 181, 207), and aliphatic acids (216).

**Position of the Hydrophilic Group.** With a given chain length, the positions of the hydrophilic groups are important variables in the determination of the surface active properties of the resultant molecule. Dreger and co-workers (39) prepared an isomeric series of sodium *sec*-pentadecanol sulfates in which the sulfate appeared successively on  $C_2$ ,  $C_4$ ,  $C_6$ , and  $C_8$ . The surface activity was found to have increased in the same order. This increased surface activity of compounds with hydrophilic groups near the center of the carbon chain has been the basis for the development of the 'tergitol' (242) and 'aerosol' series (31) of surface active agents.

When these compounds were tested for detergency and wetting ability, it was found that, other things being equal, the nearer the hydrophilic group is to the

end of a straight-chain alcohol the better the detergency but the poorer the wetting ability and vice versa. These results emphasize the limited applicability of measurements of surface activity at model interfaces such as the air-water interface (surface tension) or the hydrocarbon-water interface (interfacial tension).

As one's attention is transferred from straight chain to aromatic compounds the possibilities for alteration in substituent hydrophilic groupings increases markedly. Unfortunately, there has been a dearth of careful, systematic investigation of the relation of structure to function of these compounds.

*Electrolytes and Surface Activity.* It is well known that colloidal micelles are stabilized by an electric double layer and that the presence of added electrolytes within the solution markedly affects this stability. Those colloids that are *negatively* charged have their charge diminished by the presence of *cations* of an added electrolyte. If the concentration of the added electrolyte is high enough, the electrical charges of the micellar surfaces are diminished to the extent that their stability is destroyed, and they flocculate. In general, the flocculating power of an ion follows the Schulze-Hardy rule in that the precipitating power of an electrolyte depends upon the *valence* of the ion, the charge of which is opposite to that on the colloidal particle.

The fact that surface active agents tend to form colloidal micelles at low and somewhat indeterminate concentrations has already been commented upon. It is to be expected, therefore, that the nature and amount of the electrolytes present will have an effect upon the properties of solutions of a given surface active agent. This expectation has been found to be experimentally true. The hydrophilic portion of the anionic type of surface active agent is negatively charged. Therefore, these compounds should be sensitive to the nature and amount of the cationic component of the electrolytes present in the solution. Progressive and considerable lowering of the critical concentration and minimal interfacial tension (water-xylene) of dodecyl sulfate solutions has been demonstrated by the addition of NaCl (182). The influence of the added salts on the interfacial tension was demonstrated to be due solely to the added cation since all the sodium salts of non-complex anions gave results similar to those given by sodium chloride. In accordance with the predictions of the Schulze-Hardy rule, cited above, there is a considerable valency effect in the concentration of added electrolyte necessary to produce a given change in surface or interfacial tension. With calcium chloride, effects equivalent to those obtained with sodium chloride, require only 1/40 to 1/200 the concentration. Similar results were obtained by studying the effect of electrolytes upon the surface tension of tridecane-7-sulfate solutions (39). The valency effect of the cations was well illustrated in this work by measurement of the molar ratio of NaCl:CaCl<sub>2</sub>:AlCl<sub>3</sub> necessary to reduce the surface tension a given amount (53 to 27.4 dynes/cm). The values were found to be 54:6:1.

#### b. Cationic Surface Active Agents

In the case of the cationic surface active agents which, since the publication of Domagk (38), have found widespread use because of their bactericidal powers,

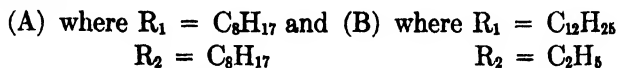
little published work exists on the relation of structure to function in terms of chemical and physical properties. The bulk of the recent literature on this subject is confined to biological activity as the main criterion of function.

While earlier sporadic publications may be found, it seems certain that the papers of Jacobs and co-workers were the first to investigate extensively the bactericidal properties of quaternary salts and the relation of their structure to function (89, 90, 91). The general discussion of chemotherapy by Jacobs (89) emphasizes bactericidal power, specificity, compatibility with tissue components, and resistance to metabolic alteration as viewpoints for evaluation of chemical compounds as potential chemotherapeutic agents. The type of compounds studied were the quaternary salts of hexamethylenetetramine coupled with benzyl halides. While the routine testing was performed on *Eberthella typhosa*, enough tests were made on other organisms to demonstrate a variability in the bactericidal powers of various compounds dependent upon the test organism used (90). In the benzyl hexamethylenetetrammonium salts, introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus notably enhanced the bactericidal powers of these compounds. Substitution in an ortho position almost invariably was more effective than substitution in the meta or para position. These classical studies in chemotherapy evidently failed to strike a responsive chord since practically no further work on quaternary salts as bactericides can be found prior to that of Domagk (38) to which reference has already been made.

*Chain Length.* Systematic studies of the structure of quaternary ammonium compounds and their bactericidal functions have uniformly emphasized the importance of carbon chain length in determining activity. Considering the simple aliphatic quaternary ammonium compounds of the general type  $RN^+(CH_3)_3$ , where R is a straight chain alkyl group, it has been found that as the chain length was increased from  $C_6$  to  $C_{18}$  the bactericidal activity increased substantially, reaching a maximum at  $C_{16}$  (84, 208). Similar conclusions of dependence of bactericidal activity of cationic surface active agents upon their carbon chain length have been derived from studies of alkyl pyridinium chlorides (111, 210), alkyl-dimethyl-sulfonium iodides (118), dialkyl-methyl-benzyl-ammonium chlorides (122), dialkyl-benz-triazolium bromides (124), azinium salts (238), sulfamyltetrazolium salts (93), alkyl-triethyl phosphonium and arsonium salts (94), alkyl-dimethyl-benzyl-phosphonium and arsonium salts (94), alkyl-colaminoformylmethyl-pyridinium chlorides (49), alkyl-phenoxy-ethoxyethyl-dimethyl-benzyl-ammonium chlorides (197), imidazolium and imidazolinium salts (211), alkyl-benzyl-ammonium chlorides, alkyl-dimethyl-ethyl-ammonium bromides, alkyl-dimethyl-allyl-ammonium bromides, and alkyl-trimethyl-ammonium bromides (232).

It might be well, at this juncture, to emphasize the fact that species differences may play a large part in the results obtained in testing chemical compounds for bactericidal activity. Not only are the minimal bactericidal concentrations quantitatively different for different species of bacteria but qualitative differences may well show up in studies of effects of alteration of chemical structure upon activity. As an example of the latter point, when the bactericidal activ-

ities of the alkyl-trimethyl-ammonium bromides were studied over the range  $C_6$  to  $C_{18}$  against *Staphylococcus aureus* and *Eberthella typhosa* it was found that with the  $C_6$ ,  $C_8$ , and  $C_{12}$  compounds *E. typhosa* was killed at higher dilutions than was *S. aureus* whereas with the  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  compounds the reverse was true (208). As another example the experience of Kuhn and Westphal with dialkyl-benztriazolium salts may be mentioned (124). In this structure (see figure 9) when one compares the bactericidal activity of the following two compounds:



the ratio of activities (A/B) is found to be 8/1 for *Staphylococcus aureus* but 1/1 for *Salmonella paratyphi* B.

*Nature of Anion.* In considering the effects of the structure of various portions of the molecules of cationic surface active agents upon their properties several investigations have been made of the effect of alteration of the anionic component of quaternary ammonium salts. Hauser and Niles (76) studied the

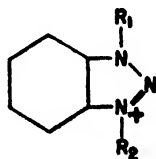


FIG. 9. DIALKYL BENZTRIAZOLIUM ION

surface tension of cetyl-pyridinium (I) chloride, bromide, and iodide and of cetyl-trimethyl-ammonium (II) chloride, bromide, and iodide. In the case of both cationic groups (I) and (II) the presence of the anionic groups enabled them to lower the surface tension of water in the order  $I > Br > Cl$ . Furthermore, the magnitude of surface tension lowering is the same for each pair of halide salts, i.e., (I) chloride and (II) chloride. This suggests that the surface tension is not dependent only upon the cation but that the nature of the anion determines its final degree.

In contrast to the physical properties of the quaternary ammonium salts, which we have seen are markedly affected by the nature of the anion, the bactericidal properties seem quite independent of the identity of the anion. It has been shown for cetyl-trimethyl-ammonium salts that the chloride, bromide, iodide, nitrate, sulfate, methosulfate, acetate, benzoate, cyanide, hydrocinnamate, and fluosilicate gave no significant differences in bactericidal power (208). The phosphate, laurate, and salicylate gave a lower activity. Similarly with cyclic compounds it was shown for the cetyl-pyridinium salts, that the nitrate, sulfate, methosulfate, bromide, and chloride have approximately identical activities (210). With acetoxy and carbethoxy derivatives of aliphatic quaternary ammonium salts the chlorides, bromides, iodides, and nitrates again were not significantly different in bactericidal activity (209).

*Nature of Central Atom.* Quaternary salts containing central atoms other than N can be formed. A series of alkyl-dimethyl-sulfonium iodides (substitution of S for N) have been prepared and their bactericidal effects against *Staphylococcus aureus* and *Escherichia coli* compared with the corresponding ammonium salts (118). The sulfonium compounds have been found to be qualitatively the same but quantitatively about one-third as active as the corresponding ammonium compounds. This work was later extended to a comparative study of quaternary ammonium, phosphonium, and arsonium compounds (94). Corresponding alkyl-triethyl ammonium, phosphonium, and arsonium iodides were compared over the range of C<sub>8</sub> to C<sub>16</sub>. Similarly, alkyl-dimethyl-benzyl ammonium, phosphonium, and arsonium compounds were compared over the same carbon chain range. The criterion of activity used was the inhibition of glycolysis of lactic acid bacteria (for method, see Jerchel (93)). For both series of compounds the relative order of activity was As > P > N.

*Electrolytes and Surface Activity.* The comments, previously made, of the effects of added electrolyte upon the properties of anionic surface active agents should be applicable to their cationic counterparts (keeping in mind, of course, their difference in electrical sign). This has been confirmed by the demonstrated effectiveness of added electrolyte in increasing the efficiency of cetyl-trimethyl-ammonium bromide both from the standpoint of surface tension lowering and bactericidal effect (81).

*Cationic Surface Active Agents of Complex Structure.* Many cationic surface active agents of complex structure have been investigated as potential bactericides. Departure from the relatively simple aliphatic quaternary ammonium salts (e.g., cetyl-trimethyl-ammonium bromide) or the simple aromatic quaternary salts (e.g., cetyl-pyridinium chloride) has not, as yet, been too fruitful.

Kuhn and co-workers have investigated the quaternary salts of aminophenol ethers (119), of hydroxyquinoline ethers (123), alkyl-triazolium compounds (124), alkyl-tetrazolium compounds (120, 121).<sup>4</sup> The compound of outstanding activity reported by these workers was the ethobromide of *n*-dodecylbenzotriazole. Dilutions of 1:615,000 were claimed to be enough to kill *Staphylococcus aureus* as compared with 1:1,200 for lauryl-dimethyl-benzyl-ammonium bromide which was used as a standard (124). These results, however, have failed to be confirmed (197, 231, 239). Varied syntheses of complex quaternary ammonium salts have been reported from the laboratory of Niederl but physical and biological data on these compounds are meager.

### c. Non-Ionic Surface Active Agents

Comprehensive reviews of methods of preparation, properties, and applications of non-ionic surface active agents have recently been published (66). Little information was found, however, which would relate the structure of these compounds and their activity.

<sup>4</sup> A brief summation of this work is presented by Westphal and Jerchel (239).



## INTERACTION BETWEEN PROTEINS AND SURFACE ACTIVE AGENTS

Studies of the interaction of surface active agents and proteins are of importance in providing a potential theoretical basis applicable to the interaction of surface active agents with other, more complex, biological systems. It has been found that these interactions may result in precipitation, complex formation, and denaturation.

*Precipitation.* Protein and surface active agents, upon interaction, may precipitate from solution. This precipitation is dependent upon such factors as the length of the paraffinic carbon chain of the surface active agent, the pH, the mass ratio of surface active agent to protein, temperature, and ionic strength (187).

The relative effectiveness of compounds in precipitating crystalline lactalbumin and egg albumin has been determined for several homologous series, such as sulfate esters of primary alcohols, sulfonates of diesters of succinic acid, and sulfonates of benzene ring compounds (144). Under constant conditions of temperature, acidity, and concentration, sulfates and sulfonates containing less than 8 carbon atoms in their paraffinic chain were ineffective precipitating agents. Increasing the length of the chain above 8 carbon atoms enhanced the precipitating effectiveness with each additional carbon until a maximum amount of precipitate per mole of reagent was reached. Further lengthening of the carbon chain was without effect. It may be well to point out that compounds with paraffin chains of 8 carbons or less do not have a marked tendency to form micelles (e.g., see figure 7). This increased efficiency of the higher molecular weight sulfates and sulfonates as protein precipitants is similar, in outline, to the increased affinities of these same anions for wool proteins as demonstrated in extensive titration data (220, 221).

With surface active agents of carbon chain length appropriate for effective precipitation, it has been reported that cationic surface active agents precipitate protein only at pH values alkaline to the isoelectric point of the protein (117). Conversely, anionic surface active agents have been found to precipitate protein only on the acid side of the isoelectric point of the protein (167, 187). Indeed, it has been suggested that rough estimations of the isoelectric point of a protein may be made by noting the lowest pH at which the protein is precipitated by a cationic surface active agent<sup>5</sup> (92).

With other factors kept constant, the mass ratio of surface active agent to protein has been found to be a decisive factor in precipitation (187). In their studies of the dodecyl sulfate-horse serum albumin system, Putnam and Neurath (187), keeping the protein concentration constant and varying the concentration of surface active agent, found that they could distinguish three separate regions. In the first region, that of protein excess, the weight ratio of surface active agent

<sup>5</sup> While this paper confines its discussion to estimation of the isoelectric point by determination of the most acid pH at which a cationic surface active agent will cause precipitation, there is no reason why the converse situation, determination of the most alkaline pH at which an anionic surface active agent will cause precipitation, could not be used equally well.

to protein is less than 0.2 and the protein is incompletely precipitated. In the second region, the equivalence zone, the weight ratio of surface active agent to protein falls between 0.2 and 0.4 and complete precipitation of the protein is accomplished. In the third region, that of surface active agent excess, the weight ratio of surface active agent to protein exceeds 0.4 and any precipitate initially formed may be partly or completely dispersed on shaking. This effect of high concentrations of surface active agents has been generally observed and, indeed, can prevent the precipitation of denatured protein at the isoelectric point or the precipitation of protein denatured by trichloroacetic or tungstic acid (5).

*Complex Formation.* While the interaction of proteins and surface active agents to form precipitates has been shown to be limited to regions in which these compounds bear charges of opposite sign, it should not be inferred that there is no interaction when both are similarly charged. Studies of such physical properties as electrophoresis, viscosity, and diffusion have demonstrated that interaction and complex formation may occur when both reactants bear the same net charge (e.g., an anionic surface active agent forming a complex with a protein on the alkaline side of its isoelectric point) (135, 160, 186, 188, 189). Electrophoretic studies (135) of the interaction of crystalline egg albumin and an anionic compound, alkylbenzene sulfonate, at pH 6.5 have demonstrated that up to a weight ratio of surface active agent to protein of 0.3 a complex is formed, the composition of which remains constant at 1 part by weight of surface active agent to 3 parts by weight of protein, and any excess of protein migrates as a separate boundary. When the weight ratio of surface active agent to protein exceeds 0.3 the electrophoretic mobility is proportional to the composition but intermediate between the mobilities characteristic of the protein and surface active agent. Similarly, the interaction at pH 6.8 of crystalline horse serum albumin and the anionic compound, dodecyl sulfate, has resulted in the formation of two discrete complexes, the electrophoretic mobilities of which are intermediate between those for the dodecyl sulfate and the horse serum albumin (189). The first complex corresponds to a weight ratio of 0.22 grams sodium dodecyl sulfate per gram of albumin while the second complex corresponds to a weight ratio of 0.45. Any excess surface active agent migrates as an independent boundary. Viscosity and diffusion studies of the same system confirm these results (160).

Reference to our discussion of precipitation will show that the minimum and maximum weight ratios of surface active agent to protein for complete precipitation of horse serum albumin by sodium dodecyl sulfate are identical with the weight ratios required for the formation of the two complexes observed electrophoretically.

*Denaturation.* We have seen, thus far, that interaction of surface active agent with protein may, with appropriate mass ratios of reactants, result in the formation of discrete stoichiometric complexes. In the pH range in which the components are oppositely charged precipitation may occur. Complex formation is, however, independent of pH over a wide region.

In addition to precipitation and complex formation, surface active agents have a powerful denaturing effect upon proteins (5). Hemoglobin and egg albumin,

at their isoelectric points, were found to be denatured by surface active agents with the denatured isoelectric protein kept in solution.<sup>6</sup> The most striking observation in these experiments was the relatively minute amount of surface active agent necessary to accomplish denaturation. For example, in the denaturation of beef methemoglobin in neutral solution, 0.0008 M duponol PC<sup>7</sup> denatured rapidly, in contrast to 8 M urea which took a considerable amount of time.

Egg albumin, a protein upon which many previous studies of denaturation have been reported, demonstrates the same proportion of sulfhydryl groups liberated by duponol PC as by guanidine hydrochloride (6). Expressed as cysteine, the sulfhydryl groups liberated in egg albumin denatured by either of the above compounds is 1.24 per cent which may be compared with the figure of 1.41 per cent reported by Hess and Sullivan (80) as the total cysteine content of egg albumin. The maximum proportion of sulfhydryl groups liberated by the surface active agent from egg albumin, 1.24 per cent, exceeds that liberated by urea, 0.96 per cent (154), or heat coagulation, 0.58 per cent (155). The latter two methods evidently fail to liberate the total number of sulfhydryl groups in the protein.

While the liberation of certain reactive groups, which are initially inert, is a widely studied manifestation of protein denaturation, there are other chemical, physical, and biological differences between native and denatured protein which have been demonstrated as a result of interaction with surface active agents. Solutions of globular proteins possess a higher viscosity in the presence of neutral denaturants, thought to be due to an unfolding and consequent greater asymmetry of the protein molecule (159). Both cationic and anionic surface active agents produce a large increase in the relative viscosity of serum albumin which is independent of pH but varies with the mass ratio of surface active agent to protein (160). In agreement with the methemoglobin experiments cited previously, these viscosity studies demonstrate that surface active agents produce changes comparable to those produced by other neutral denaturants but at much lower concentrations. Thus, similar alterations in the intrinsic viscosity of serum albumin are brought about by 8 M urea and 0.17 M sodium dodecyl sulfate, by 8 M guanidine hydrochloride and 0.28 M sodium dodecyl sulfate (160). Some of the other manifestations of protein denaturation which have been observed as a result of the presence of surface active agents include: a less ready crystallizing tendency of the protein after removal of the denaturant (187), alteration of x-ray diagram, indicating change from corpuscular to fibrous arrangement (166), alteration of molecular weight (153, 215), and loss of specific biological activity (13, 14, 215). Serum proteins regenerated after denaturation by urea or guanidine have been shown to retain the immunological specificity characteristic of the native protein, although possessing a decreased antigenic activity (50, 150). However, similar studies with proteins denatured by surface active agents are lacking.

<sup>6</sup> This solubilization of the denatured protein is valuable in enabling titration of the liberated sulfhydryl groups in homogeneous media.

<sup>7</sup> A mixture of the C<sub>10</sub> to C<sub>14</sub> alcohol sodium sulfates.

It will be noted that the discussion of the interaction of surface active agents and proteins has, thus far, been limited to the ionized agents. This is natural since the phenomena which we have reviewed have been primarily the consequences of electrostatic interaction. However, it should not be forgotten that another type of surface active agent remains: the non-ionic. Relatively little data exist in the literature concerning the interaction between this type of compound and proteins. No evidence could be found by electrophoresis (134) for any interaction or complex formation between either native or heat denatured egg albumin and a polyether alcohol. These results are consonant with the hypothesis of strong polar groups in surface active agents being requisite to interaction with proteins. This lack of interaction of non-ionic surface active agents and proteins has important consequences since it allows their coexistence in solution with maintenance of altered physical characteristics such as lowering of surface tension, solubilization, etc., but divorced from such biologically deleterious effects as denaturation.

#### INTERACTION BETWEEN SURFACE ACTIVE AGENTS AND ISOLATED BIOLOGICAL SYSTEMS

##### *Enzymes*

Studies of the interaction of surface active agents and enzymes are meager. In common with other proteins, such enzymes as pepsin, urease, yellow ferment, and catalase are precipitable only when their electrical charge is opposite in sign to that of the surface active agent (117, 187). The activity of urease and phosphatase has been found to be only slightly inhibited by alphasol Ma<sup>8</sup> (148), but, since the enzyme concentrations were not given and only a single concentration of surface active agent was used, evaluation of these results is difficult. Freeman *et al.* (55) have demonstrated that complete inhibition of enzymatic activity of amylase, lipase, and pepsin could be effected by an anionic surface active agent (an alkyl-aryl sulfonate, not further identified) while partial inhibition was obtained with trypsin and phosphatase. In agreement with the low concentrations of surface active agent previously shown to be sufficient for denaturation, those complete inhibitions require solutions of only 0.01 M or less surface active agent.

The activity of crystalline trypsin can be diminished by interaction with soaps, and complete inhibition is achieved in the presence of sufficient soap (169). Soaps of the same paraffin chain length (C<sub>18</sub>) but differing in the number of double bonds were equal in their ability to inhibit tryptic activity. Partial reversibility of this inhibition occurred when the soap was precipitated from the soap-enzyme-substrate mixture by addition of calcium chloride. It can be calculated from the data presented in this paper that the mass ratio of soap to protein necessary for complete inhibition approximates 2.5.

In what may well be a process analogous to the dissociation of conjugate proteins, anionic surface active agents can activate protyrosinase to produce active tyrosinase (3, 24). When an homologous series of alkyl sulfates was tested,

<sup>8</sup> Dihexyl sodium sulfosuccinate.

optimal activating efficiency was found in the 12 to 16 carbon range. Attempts to reverse this activation by removal of the surface active agent from the field of action have not been successful (25). Cationic surface active compounds, however, were not effective as protyrosinase activators (4).

The absence of interaction between non-ionic surface active agents and protein has been mentioned previously. Advantage has been taken of this situation to formulate a method for the determination of lipase activity (7). Utilizing as a substrate the non-ionic compound, tween 20,<sup>9</sup> which is completely soluble in water, lipase activity can be determined in a one-phase system instead of the two-phase emulsion previously required.

### *Toxins*

Bacterial exotoxins have properties which have, for a long time, caused them to be identified with proteins. This hypothesis has been greatly strengthened by the isolation, in recent years, of several toxins as essentially pure, highly active proteins (diphtheria (47, 168), tetanus (176), botulinus, type A (1, 126), and botulinus, type B (125)). It is reasonable to expect, therefore, that these toxins upon interaction with ionic surface active agents, will be subject to alterations similar to those exhibited by other proteins. Like enzymes, toxins furnish a potent biological activity for measurement in addition to the physical properties susceptible to measurement in other proteins.

Tetanus toxin has been inactivated by bile salts and soap (131, 132, 234) and by the cationic compound zephiran<sup>10</sup> (158). Similarly, diphtheria toxin may be inactivated by soaps (17, 19, 132) and the salts of fatty acids (205). The lower members of the fatty acid series produced little inactivation, but, beginning with the acid containing 8 carbon atoms in the paraffinic chain, a destruction of toxic, flocculating, and immunizing properties is produced by solutions as dilute as 0.01 M. The lecithinase of *Clostridium welchii*, which is probably identical with the specific alpha toxin, is readily inactivated by sodium dodecyl sulfate (142).

While it has been claimed that tetanus and diphtheria toxins detoxified by sodium ricinoleate are excellent antigens (130), contrary results have also been obtained (205).

### *Erythrocytes*

Surface active agents, in contact with biological cells, frequently cause cytolysis. This has been long recognized in conjunction with red blood cells where the hemolytic effect of such natural surface active compounds as the bile salts and saponins have been the subject of many investigations (179). Since the introduction of synthetic surface active agents, several observations of their hemolytic properties have been recorded (5, 21, 83, 94, 211). The hemolytic capacity of an homologous series of anionic compounds has been found to vary with chain length (83, 180), with optimal efficiency, in the sulfated alcohols, at 14 carbon atoms,

<sup>9</sup> A polyoxyalkylene derivative of sorbitan monolaurate.

<sup>10</sup> A mixture of the C<sub>8</sub> to C<sub>18</sub> dimethyl benzyl ammonium chlorides.

where a 1:100,000 dilution is lytic within a few minutes. Plasma and its protein and lipid components are capable of inhibiting these hemolytic effects.

At sub-lytic concentrations, surface active agents still produce changes as evidenced by alteration of erythrocyte shape from discoidal to spheroidal. These disk to sphere transformations may be produced at surface active agent concentrations only 1/10 of that necessary for hemolysis. For the most efficient alcohol sulfates, calculation of erythrocyte surface area per molecule of lysin shows that spherizing may be accomplished even though there are not sufficient molecules of lysin in the system to cover the erythrocyte surfaces with a continuous monolayer (180).

Within a restricted number of homologous anionic compounds, a relationship between surface activity and hemolytic activity is found (83). When the scope of the chemical structures tested is broadened, however, this relationship no longer holds true. Ionic compounds with like surface activity are found to have widely different hemolytic activities and certain non-ionic compounds are found to be non-hemolytic at concentrations which demonstrate surface tension properties equivalent to those of anionic and cationic compounds which are intensely hemolytic (65). This lack of hemolytic activity by non-ionic compounds lends support to the view of Ponder (180) that surface active agents form complexes with lipid, lipoprotein, and protein components of the erythrocyte ultrastructure as a stage in the hemolytic process. That this interaction of surface active agents and erythrocytes may operate to alter the normal ionic permeability of the cellular membrane to produce a so-called "colloid osmotic hemolysis" has recently been indicated (240, 241).

Many of the above mentioned experimental observations on the interaction of erythrocytes and surface active agents and the theoretical inferences as to their mechanism will find analogies in the coming discussion of the interaction of bacteria with similar compounds.

### *Bacterial Growth*

Alteration of surface tension may produce marked effects on the growth of microorganisms. The bulk of the studies that were completed before the advent of modern synthetic surface active agents utilized such substances as soaps, bile salts, and saponin as surface tension depressants (for literature of this early work see 56, 133).

It is well to recognize that culture media ordinarily have surface tension values lower than that of water, due to their protein content. Thus Marshall (149), investigating a number of routinely used culture media, found their surface tensions in the order of 45 to 54 dynes/cm as compared with 72 for water.

Reducing the surface tension of the medium below 45 dynes/cm may well change the character of growth of microorganisms. In media of low surface tension, *Bacillus subtilis* and *Mycobacterium tuberculosis* may cease pellicle formation and grow submerged and dispersed throughout the bulk of the liquid, and some anaerobes, particularly *Clostridium tetani*, have been reported to grow aerobically (128, 129). It is difficult to ascribe this last effect to surface activity

and, as a matter of fact, doubt exists as to the accuracy of the observation (64). In addition to alteration of the character of growth, culture media of low surface tension may depress or prevent growth. Pneumococci<sup>11</sup> and streptococci have been shown to grow poorly in media, the surface tension of which was lower than 50 dynes/cm (9, 127, 178) while 46 dynes/cm has been found as a limiting surface tension for the growth of *Bacillus anthracis* (127). Growth of *Mycobacterium tuberculosis*, human type, was inhibited at surface tensions of 42 dynes/cm while the avian type and *Mycobacterium phlei* were inhibited at surface tensions below 30 dynes/cm (2). Bacteria flourishing in the gastrointestinal tract seem to be resistant to the deleterious action of surface active compounds in their culture media (127, 218, 245). The following order of sensitivity of growth to depression of surface tension has been determined by Wolf (245). The list commences with the most sensitive.

*Corynebacterium diphtheriae*  
*Clostridium welchii*  
*Erysipelothrix murisepticus*  
*Bacillus subtilis*  
*Clostridium sporogenes*  
*Bacillus proteus*  
*Eberthella typhosa*  
*Pseudomonas pyocyaneus*  
*Escherichia coli*  
*Salmonella paratyphosus A*  
*Salmonella paratyphosus B*

It is well to consider these early experiments on the relation of surface tension to bacterial growth with a good deal of reserve. Most of the observed effects are probably more ascribable to specific effects of the surface active agent used than to alteration of surface tension *per se*. As was seen in our discussions of the interaction of surface active agents with proteins, or with erythrocytes, the ionic nature of the surface active compound is an important factor in the results obtained. Non-ionic compounds do not interact with proteins even though they possess surface tension depressant properties similar to the ionic compounds which, under similar circumstances, may cause precipitation, complex formation and denaturation. With the erythrocyte, too, some non-ionic compounds were non-hemolytic despite surface tension alterations which, in the case of the ionic compounds, were far above the minimum necessary for complete hemolysis. That similar factors are of importance in determining the results of the interaction of surface active agents and bacterial cells may be concluded from the more recent work, which shows that, dependent upon the nature of the surface active agent used, a whole gamut of results may be obtained from bactericidal activity at the one extreme to facilitation of submerged, disperse growth at the other.

Studies of the effects of a variety of cationic and anionic surface active agents on the respiration and glycolysis of both gram positive and gram negative micro-

<sup>11</sup> Synthetic surface active agents will cause pneumococci to lyse (18) and may be used instead of bile salts in the differentiation of pneumococci from streptococci (73).

organisms have demonstrated the superior effectiveness, at physiological pH, of the cationic compounds as inhibitors<sup>12</sup> of bacterial metabolism (10). While the cationic compounds are equally effective against both gram positive and gram negative organisms, the anionic compounds show a selective activity against the gram positive organisms. A similar selectivity is illustrated in the effects of anionic compounds on the lactic dehydrogenase and cytochrome systems of suspensions of *Staphylococcus aureus* and *Escherichia coli* (163). As one departs from physiological pH the cationic compounds become more active in the alkaline range, and the anionic in the acid range. In common with the previously discussed effects of chain length on the activity of surface active agents, maximal effectiveness for inhibition of bacterial metabolism has been demonstrated in the C<sub>12</sub> to C<sub>16</sub> range.<sup>13</sup>

An outstanding current application of surface active agents in bacteriology has been the discovery of the effect of tween 80, a non-ionic, fatty acid ester type compound<sup>14</sup>, in promotion of submerged and diffuse growth of tubercle bacilli (44). Although virulent tubercle bacilli are among the least fastidious of pathogenic microorganisms in their metabolic requirements, *in vitro* growth of these organisms has necessitated relatively large inocula and long periods of incubation with all the disadvantages inherent in the resultant heterogeneity of the cellular population.

Utilizing a modified Kirchner medium Dubos and Davis have demonstrated that addition of tween 80, up to an optimal level of 0.1 per cent, greatly enhanced the rate and abundance of growth of an avian (Kirchberg) strain. Corresponding experiments with a human strain (H37RV) are more difficult to assess from the standpoint of the amount of growth due to the granular masses of the control cells as compared with the isolated cells and microscopic loose clumps prevailing in the culture grown in the presence of tween 80. This ability to facilitate submerged and diffuse growth of mycobacteria in liquid media seems resident in the surface active properties of the substance since non-surface active oleic acid esters are not effective.

While tween 80 promotes the diffuse submerged growth of tubercle bacilli, early experiments required relatively large inocula. This inhibitory effect against small inocula has been found to be due to small amounts of unesterified oleic acid<sup>15</sup> present either in the original commercial tween 80 or formed as a result of hydrolytic action of biological material on this product. Chemical

<sup>12</sup> At concentrations below those effective in causing inhibition of bacterial metabolism an actual stimulation of metabolism may, sometimes, be found (10). This stimulatory effect has been found much more frequently with anionic than with cationic surface active compounds.

<sup>13</sup> Growth retardation and inhibition may, however, occur at concentrations of surface active agent which have little or no effect on cellular energy production (201).

<sup>14</sup> A polyoxyalkylene derivative of sorbitan monooleate.

<sup>15</sup> Although free long chain fatty acids may be inhibitory to many microorganisms, the same compounds esterified or formed into complexes with native serum albumin may have these toxic effects minimized or eradicated and the resultant atoxic compounds may actually enhance the growth of certain bacteria (43).



extraction of this unesterified fatty acid (35) or its removal from the field of action by complex formation with such substances as serum albumin<sup>18</sup> allows successful growth from truly minimal inocula of 2 or 3 cells (36, 37).

Tubercle bacilli grown diffusely in submerged liquid culture in the presence of tween 80 retain their characteristic morphology and staining (45). This has been true even in cultures maintained for over a year with repeated transfers in liquid media containing this product. Return of these cultures at any time to standard media (e.g., egg yolk media) causes reversion to a granular type of growth. In addition to retention of characteristic morphology and staining, pathogenic mycobacteria grown in the presence of tween 80 are extremely virulent for mice, guinea pigs, and chick embryos and are able to elicit the production of agglutinins for the homologous cultures. It is well to caution, however, that tween 80 in conjunction with the tubercle bacilli may act as a haptene, thus serving as a potential source of confusion in immunological analysis.

Successful applications, using both fluid and solid media incorporating this surface active agent, have been made to studies of the pathogenesis of experimental tuberculosis in mice (174), the morphological characteristics correlated with the virulence of mammalian tubercle bacilli (151), the rapid cultivation of tubercle bacilli from pathological material (54), the demonstration of naturally occurring streptomycin resistant variants of the tubercle bacillus (233), and the revelation of the antibiotic activity of subtilin against *Mycobacterium tuberculosis* (247).

Stimulated by the obvious advantages of having water-soluble non-toxic solutions of oleic acid in the form of tween 80, Williams *et al.* (243) have made use of this compound in studying the metabolism of lactic acid bacteria.

#### *Bacteriostatic and Bactericidal Activity*

Current interest in the application of surface active agents, other than soaps, as bactericides stems from the work of Domagk (38) although scattered previous indications of the bactericidal possibilities of these compounds is available in the literature (75, 89, 90, 91). We have already discussed the role of such factors in chemical structure as alkyl chain length, nature of the anion, nature of the central atom, and biological specificity in determination of the bactericidal properties of quaternary ammonium salts.

Since it has been widely observed that in any homologous series of surface active compounds the bactericidal efficiency and the surface depressant properties increased with increasing alkyl chain length, it has been natural to consider the possible relation between surface activity and bactericidal activity. While all surface active compounds which are efficient bactericides have been found to possess a marked ability to reduce surface tension, the converse is not necessarily true. Extensive experimental evidence of this may be found in surface tension determinations on a series of salts of high molecular weight aliphatic acids which had been previously studied for their bactericidal effects on *Mycobacterium*

<sup>18</sup> Even purified serum albumin may contain some lipase which may be inactivated by heating at 56 C.

*leprae* and other acid-fast bacteria (216, 217). Similar conclusions have been reached from a study of various commercial surface active compounds (59). Further confirmation may be inferred from the demonstration that non-ionic surface active agents, although effective as depressants of surface tension have little or no effect on bacterial metabolism and are not bactericidal (12, 85).

In our discussion of the inhibitory effects of surface active agents on bacterial metabolism we saw that cationic compounds, at physiological pH, are more effective than anionic (10). In addition, cationic compounds possess equal effectiveness against both gram positive and gram negative organisms while the anionic compounds show a selective activity against gram positive organisms. In general, the results of studies of inhibition of bacterial metabolism and bactericidal activity of surface active agents parallel each other, although quantitative correlation has not always been obtained (11)<sup>17</sup>. With respect to the selective activity of anionic and cationic surface active compounds, similar conclusions may be drawn from studies of their bactericidal activity<sup>18</sup> as were obtained from studies of their inhibition of bacterial metabolism (11, 23, 33).

In these comparative studies of the effects of surface active agents on bacterial metabolism and viability, some interesting observations were made on a non-quaternary type of cationic compound (10, 11). These compounds are alkyl esters of amino acids (e.g., the lauryl ester of alpha-amino isobutyric acid). When they possess an optimal number of carbon atoms in the amino acid they are effective in inhibiting bacterial metabolism but quite ineffective as bactericides. No explanation of this discrepancy is apparent. Unfortunately, there seems to be no other published work on the biological activity of these compounds to which these results can be compared.

As one departs from a neutral pH, the cationic compounds tend to become more efficient bactericides in more alkaline solutions and less efficient in acid solutions with the reverse situation holding true for the anionic compounds (48, 59, 60, 61, 84, 190). Cetyl pyridinium chloride, however, seems to maintain an even level of bactericidal activity over the pH range of 2 to 10 (190) with no explanation apparent for its individuality in this respect. This dependence of the bactericidal efficiency of surface active agents on pH is analogous to the relationship obtaining when inhibition of bacterial metabolism is used as a criterion of activity (10) and has many points of similarity to the effects of pH on the bactericidal activity of acidic and basic dyes (219). This pH dependence also means that the relative bactericidal superiority evidenced by cationic compounds when compared, at neutral pH, with anionic compounds may not only change quantitatively as the pH is altered but, under appropriate conditions, anionic compounds may display a marked superiority over cationic. Table 4

<sup>17</sup> This may be largely due to the methods involved. In studying inhibition of bacterial metabolism one measures the actual per cent inhibition as compared with the control, whereas in bactericidal studies the presence or absence of viable cells is measured without any intermediate possibilities.

<sup>18</sup> However, in the bacteriostatic range a greater activity against gram positive bacteria may be shown by cationic compounds (107).

with figures selected from data of Gershenfeld and Milanick (59) illustrates this by a comparison between aerosol OT (an anionic compound) and triton K-12 (a cationic compound).

In common with other chemical bactericides, the efficiency of surface active agents is affected by the presence of organic matter. Whereas, under standardized experimental conditions, cetyl trimethyl ammonium bromide displays a phenol coefficient of 1200 in the absence of added organic matter, this figure decreases to 380, 225, 150, and 62 respectively in the presence of 2, 5, 10, and 20 per cent added horse serum (84). Further illustration of the effect of the presence of protein material upon the bactericidal efficiency of cationic compounds against a variety of microorganisms may be found in table 5. When one keeps in mind the complexes formed by the interaction of proteins and surface active agents this inhibition of bactericidal activity by the presence of added protein is not at all surprising. In addition to proteins, the lipids display

TABLE 4  
*Bactericidal activity of an anionic and cationic surface  
active compound at varying pH*  
Adapted from (59)

COMPOUND	MAXIMAL DILUTIONS KILLING STAPHYLOCOCCUS AUREUS IN 10 MINUTES BUT NOT IN 5, AT 37 C					
	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Aerosol OT.....	1:37,500	1:32,500	1:5,000	<1:100	—	—
Triton K-12.....	<1:100	1:150	1:400	1:900*	1:10,000	1:20,000

\* This test performed at pH 7.2.

a marked ability to counteract the bactericidal effects of surface active compounds (58). Phospholipids added before or simultaneously with surface active agents decrease or prevent the inhibitory effects on bacterial metabolism displayed by surface active compounds (12). Similar protection by phospholipids is evidenced when the bactericidal activities of surface active compounds are studied. Since the phospholipids possess a characteristic polar-nonpolar structure, they are surface active. Although they do not by themselves alter bacterial metabolism it is conceivable that, by adsorption to the bacterial surface, they alter its nature. Indirectly, this view is supported by such evidence as the retention of the protective effects of phospholipids by bacteria which have been exposed to these compounds and then washed. Then too, phospholipids added subsequent to the surface active agent are without protective effect (12). Ordinary fats, such as butterfat, (possibly due to their phospholipid content), have been demonstrated to protect bacteria against the bactericidal activity of surface active agents (194).

*Methods of Evaluation.* While there is little doubt that certain surface active agents, as represented by the quaternary ammonium and pyridinium salts, are highly efficient bactericides, concern has been expressed as to the validity and

significance of some of the high phenol coefficients reported in the literature. This concern has been engendered both by thoughts of the general inapplicability of the phenol coefficient test to determination of the bactericidal efficiency of non-phenolic compounds which may be totally unlike phenol in chemical and bactericidal properties (27, 28, 100, 198) and, more specifically, by reason of

TABLE 5  
Germicidal activity of cetyl pyridinium chloride aqueous solution  
from Quisno and Fotor (190)

ORGANISM	NO. STRAINS TESTED	AVERAGE CRITICAL KILLING DILUTION IN TERMS OF ACTIVE INGREDIENTS AT 37 C*	
		No serum	10 % bovine serum
<i>Staphylococcus aureus</i> .....	5	1:83,000	1:12,500
<i>Staphylococcus albus</i> .....	1	1:73,000	1:12,000
<i>Streptococcus viridans</i> .....	1	1:42,500	1:12,000
<i>Streptococcus hemolyticus</i> .....	2	1:127,500	1:17,000
<i>Neisseria catarrhalis</i> .....	2	1:84,000	1:13,000
<i>Diplococcus pneumoniae I</i> .....	1	1:95,000	1:14,000
<i>Diplococcus pneumoniae III</i> .....	1		1:20,000
<i>Pseudomonas aeruginosa</i> .....	2	1:5,800	<1:1,000
<i>Klebsiella pneumoniae</i> .....	2	1:49,000	1:5,500
<i>Corynebacterium diphtheriae</i> .....	1	1:64,000	1:14,000
<i>Mycobacterium phlei</i> .....	1	1:1,500	1:1,000
<i>Eberthella typhosa</i> .....	5	1:48,000	1:3,000
<i>Escherichia coli</i> .....	2	1:66,000	<1:1,000
<i>Proteus vulgaris</i> .....	2	1:34,000	1:2,000
<i>Shigella dysenteriae</i> .....	1	1:60,000	1:5,000
<i>Shigella paradysenteriae</i> (Flexner).....	2	1:52,000	1:3,500
<i>Shigella paradysenteriae</i> (Hiss).....	1	1:49,000	1:2,000
<i>Shigella sonne</i> .....	2	1:68,000	1:6,500
<i>Lactobacillus acidophilus</i> .....	1		1:16,500
<i>Brucella abortus</i> .....	1		1:19,500
<i>Trichomonas vaginalis</i> .....	1		1:3,000†
<i>Candida albicans</i> .....	1	1:37,000	1:3,500
<i>Cryptococcus neoformans</i> .....	1	1:61,000	1:6,000
<i>Trichophyton mentagrophytes</i> .....	1	1:36,000	1:3,000
<i>Microsporium canis</i> .....	1	1:34,000	1:5,000

\* The critical killing dilution is defined as the highest dilution of germicide which will kill in 10 minutes but not in 5.

† Twenty-five per cent human serum.

the individualistic properties of cationic surface active agents (22, 103, 104, 199). Factors in the Food and Drug Administration method of testing germicides (203) against which criticisms have been leveled include the following features of especial interest in testing surface active agents:

- Inconstancy of the culture medium.
- Failure to distinguish between bacteriostatic and bactericidal action.
- Lack of uniformity in size of transfer in preparing subcultures.
- Presence of organic matter.

Variability of the peptone component of the medium used in determination of the phenol coefficient may have serious consequences in the results obtained (26, 190, 200, 248). Beyond the obvious metabolic importance of the identity and reproducibility of the peptone content, it is important to consider the variable amounts of phospholipids possible in peptones prepared from natural materials. Phospholipids, as previously stated, are capable of interfering with bactericidal activities of cationic surface active agents (12). Recognition of this problem has caused synthetic and semi-synthetic media to be recommended (102, 246).

Distinction between bactericidal and bacteriostatic effects represents another problem which the cationic surface active agents share with other bactericidal compounds of high efficiency, such as the heavy metal salts. Methods based both on dilution and the presence of specific antagonists have been devised to meet this problem. Subculturing of organisms, which have been subjected to the action of the experimental bactericide, into large volumes of culture media or even retransferring a loopful of the first subculture into a second tube of culture medium has been suggested to minimize, by dilution, the effect of any surface active agent carried over (103, 212). Neutralization of any surface active agent carried over in subculturing may be accomplished by incorporation of lecithin (193) or sodium stearate (183) into the subculture medium. A semi-solid broth containing lecithin has also been suggested (8) which allows discrete colony growth and detection of partial inhibition.

Since the presence of surface active agents in the medium alters the surface tension, the standard loopful used in subculturing will contain varying volumes of media (and therefore varying numbers of organisms). A volumetric transfer has been recommended (183, 226).

It is universally agreed that the presence of organic matter lowers the efficiency of bactericides. It is important to keep in mind, however, that, due to such factors as the concentration, reactivity, and adsorbability of the bactericide, the presence of a given amount of organic matter may affect the efficiency of chemically different types of bactericides to varying degrees. The cationic surface active agents are used in low molar concentration so that removal of a given number of molecules from the field of action will have a proportionately greater effect upon them than on many bactericides of lower efficiency (101). The presence of phospholipids (12) and fats (194) inhibits the bactericidal activity of cationic surface active agents, in addition to proteins forming complexes with them. The high degree of surface activity of these compounds while advantageous from the standpoint of facilitating tissue penetration is disadvantageous in increasing the tendency of surface active compounds to be adsorbed out of solution. The effect of the presence of 10 per cent bovine serum on the average critical killing dilution of cetyl pyridinium chloride on a variety of microorganisms is illustrated in table 5.

An interfering factor of obvious importance in testing bactericides, but one difficult to evaluate quantitatively, is the tendency for organisms exposed to quaternary ammonium compounds to agglomerate and to adhere to the walls

of the containing vessel (40, 103, 105, 140, 191). This agglomeration and adhesion leads to obvious difficulties in obtaining uniform exposure or representative sampling of organisms and is a contributing factor to the inconsistencies universally reported in the testing of quaternary ammonium compounds by the phenol coefficient method.

Modifications of the phenol coefficient test which have been recommended for use with surface active agents include the use-dilution method, where actual concentrations recommended for sanitary use are tested (146, 199), a glass slide technique utilizing a 99.9 per cent endpoint (95), a combination of the phenol coefficient test with plate count and swab count (30), a semi-micro adaptation of the phenol coefficient test in which the entire volume rather than an aliquot of the bacterial suspension-bactericide solution is cultured (103), and a filter paper transfer technique (103, 104). Agar-plate or cylinder-plate methods of testing bactericidal activity, while widely used with chemotherapeutic agents, have not been found applicable to quaternary ammonium compounds (173, 192), possibly due to the inability of ionic aggregates to pass through the agar network (173) or due to physical adsorption of the cationic compounds on the agar (192).

An attempt has been made to avoid the difficulties and inconsistencies of the *in vitro* methods of evaluation by resort to *in vivo* testing. The developing chick embryo has been used by inoculation of the chorioallantoic membrane with *Staphylococcus aureus*, treatment of the infected membrane over a period of five days with the bactericide under test, followed by culture of the membrane for estimation of the remaining organisms (68, 69). Another method utilizes a virulent strain of *Salmonella typhimurium* as the test organism and follows the procedure of the phenol coefficient test with the modification of subculturing the treated organisms by inoculation into mice rather than culture broth. End-points were determined by recovery of organisms from the heart blood of the infected mice (98). The technique of Nungester and Kempf (161), where the tail of a mouse is immersed in the bacterial culture-bactericide solution and then a portion of the tail transplanted into the abdominal cavity of the mouse, has also been applied (175). These extensive efforts in the methodology of evaluation of the bactericidal efficiency of the cationic surface active agents have yet to yield a method which is unequivocal and universally accepted by workers in the field.

If it is proposed to utilize bactericides in contact with animal tissues it is, of course, important to have a measure of the relative toxicity of the bactericide to pathogenic bacteria and to tissue. A number of workers have measured toxicity indices (i.e., ratio of bacterial toxicity to tissue toxicity), where the toxicity to bacteria has usually been determined by the phenol coefficient test and the toxicity to tissue by the effect of the bactericide upon the phagocytosis of artificially opsonized staphylococci (82, 236, 237), or by the effect on the chick embryo (63, 244). In general, the toxicity index has been found to be lower for bactericides of the surface active quaternary ammonium type than for compounds dependent upon chlorine, mercury, permanganate, alcohol, or formaldehyde for their action (235).

**Microbicidal Activity.** The activities of several commercial quaternary ammonium salts against a variety of bacteria and fungi have been reported in the literature (e.g., 46, 96, 175, 190). As an illustration<sup>19</sup> of the bactericidal and fungicidal spectrum of these compounds the activity of cetyl pyridinium chloride has been chosen as an example and tabulated (see table 5).

In addition to their utility as bactericides and fungicides, quaternary ammonium salts have been found useful as cysticides. Several cationic surface active agents have demonstrated efficiency in the destruction of water borne cysts of *Entamoeba histolytica* while anionic and non-ionic compounds were found of lesser utility (51).

**Synergistic Activity.** Surface active agents have also attracted interest in recent years because of their possibilities in potentiating or synergizing the activity of bactericides and fungicides of different chemical nature. Addition of anionic surface active compounds to phenol derivatives has, in several studies, increased their bactericidal efficiency 50 to 100 per cent (61, 164, 165, 227). Evidence has been obtained that this enhancement of bactericidal properties is primarily due to a synergistic action between the surface active compound and the undissociated phenols (164). Ordinary soaps, which are commonly used to emulsify phenolic bactericides of limited aqueous solubility, will actually tend to decrease the bactericidal efficiency of these phenolic compounds due to the alkalinity the soaps produce upon hydrolysis and the consequent decrease in the proportion of undissociated phenol present. Ordinary soaps are not usable in acid solutions due to the limited solubility of their anionic fatty acids. The synthetic anionic surface active agents, on the other hand, are soluble in acid solution and mixtures of phenolic bactericides and surface active agents can be formulated with the phenols present in the undissociated form. This allows a maximal bactericidal effect of the phenolic compound *per se* plus maximal synergistic effect of the added surface active agent. It has also been indicated that some synergistic effects can be demonstrated between surface active agents and mercurials, permanganate, and hexylresorcinol<sup>20</sup> (53, 61).

While discussing the synergistic effects of surface active agents used in conjunction with bactericides, it may be well to caution against the formulation of mixtures of anionic surface active agents and quaternary ammonium salts. Since the quaternary ammonium salts are cationic compounds they will combine electrostatically with the anionic surface active agents and may even precipitate out of solution, with a loss of bactericidal efficiency rather than the desired increase.

**Theory of Antibacterial Activity.** Certain speculations as to the probable mechanism of the high degree of antibacterial activity of surface active agents have been made. Perhaps the first of the series of processes involved in the interaction of surface active agents and bacteria is a reversible adsorption or

<sup>19</sup> Quaternary ammonium compounds, however, are not very effective in killing spores (41).

<sup>20</sup> This synergistic effect was not demonstrable when pH changes were not carefully controlled (62).

combination of ions of these compounds with bacteria. Evidence, by analogy, for this view may be obtained by reference to the demonstrated ability of bacteria to adsorb hydrogen ions and other cations from solution and to enter into ion exchange (137, 138). Further evidence is obtained from the dependence of the bactericidal activity of surface active agents on pH which operates to increase the activity of the surface active compound when the production of oppositely charged ions in the bacterial cell is favored (231). More directly applicable is the demonstration that the inhibitory action on bacteria of cationic surface active agents may be delayed by prior treatment of the bacteria with anionic surface active agents (42). Indeed, within certain time limits, this inhibitory action may be reversed by subsequent introduction of an anionic compound (231); in addition, there is reported a protective action on bacteria of the presence of a cationic compound, which is bactericidally relatively ineffective, in conjunction with an effective cationic compound. Presumably, this protective effect operates through shifting the adsorption equilibrium of toxic cation and bacteria through competition for reactive sites.

Recent work, however, has cast some doubt on the validity of these observations (107). Anionic compounds added after the exposure of bacteria to cationic compounds were shown not to be able to reverse the bactericidal effect. Repetition of the demonstration of competitive action of bactericidally ineffective versus effective cationic compounds was not attempted. While there seems to be little doubt of the ability of bacteria to act as ion exchangers and to adsorb ions of surface active compounds from solution, the role of this mechanism in the bactericidal activity of these compounds has yet to be clearly delineated experimentally.

One of the observations in studies of the effects of surface active agents on bacterial metabolism has been an upper limit to the percentage inhibition obtained (80 to 95 per cent) regardless of increase in concentration of surface active agent (10, 11). These data have been interpreted as evidence against the hypothesis that an enzyme of key importance exists which is very sensitive to surface active agents, since it might be expected that increasing the amount of such agent would give an excess capable of totally blocking metabolic activity (85).<sup>21</sup> As an alternative consideration, the possibility of disruption of some cellular membrane component with a consequent increase in permeability is suggested—a mechanism reminiscent of that associated with erythrocyte hemolysis. With this mechanism, intracellular constituents such as enzymes, ions, coenzymes, and metabolic intermediates would be released to the surrounding medium by the lytic action of surface active agents. This dilution of the intracellular contents would reduce to a low level the metabolic activity observed with no further effect to be expected upon addition of more surface active agent until the concentration was high enough to interfere by denaturation and inac-

<sup>21</sup> However, the possibility of alternate metabolic pathways should be considered e.g., metabolic reactions involving amino acids in which dehydrogenation reactions take place through the intermediary functions of various hydrogen acceptors, without the utilization of oxygen (206).



tivation of the enzymes present. In experimental support of this lysis mechanism of the function of surface active agents, chemical analyses have been made of the trichloroacetic acid soluble phosphorus and nitrogen compounds released in the washings of staphylococci in the presence of various anionic, cationic, and non-ionic surface active agents (85). Whenever the nature and concentration of the surface active agent are adequate to be bactericidal, a leakage of nitrogen and phosphorus compounds from the cells is observed (85, 86). Confirmation of this lytic mechanism is found in studies of the release of specific amino acids from bacterial cells (57). Determinations of the release of lysine from *Streptococcus faecalis* showed that surface active agents (tyrocidin, CTAB, and aerosol OT) liberate lysine proportionate to the concentration of surface active agent used up to a level that is sufficient to liberate the total lysine of the bacterial cells. This lytic effect is quantitatively similar to the bactericidal effect of the same compounds. Non-surface active bactericides (penicillin,<sup>22</sup> acriflavin, sulfa drugs do not exhibit this lytic effect).

The two theoretical considerations presented above have emphasized the role of the bacterial surface in determining the results of interaction of surface active agents and bacteria. Within the bacterial cell there exist many compounds essential to metabolism and growth which are susceptible to interaction with surface active agents and consequent alteration of their specific metabolic activity. The ability of surface active agents, at concentrations of the same order of magnitude as were bactericidal, to precipitate proteins and, in the case of conjugated proteins, to cause dissociation, has impressed several workers (12, 117) as a possible mechanism of their antibacterial activity. This view has been challenged (85) on the basis of the quantitative differences in mass ratio of protein to surface active agent required for denaturation of ordinary proteins as compared with bactericidal effects. In view of the meagerness and roughness of the data thus far available, it would seem premature to disregard denaturation in theorizing on the mechanism of the antibacterial activity of surface active agents.

#### *Interaction with Viruses*

To the extent that virus activity may be considered resident in a protein structure, it might be expected that the conclusions drawn from studies of the interaction of proteins and surface active agents would be applicable to the interaction of viruses and surface active agents. In a limited way, this has been demonstrated with plant viruses (172). The bulk of the work on interactions of viruses and surface active agents, however, suffers both from the nature of the virus preparations and the mode of attack. Virus preparations, especially animal viruses prepared from tissue homogenates and chick embryo fluids, and protein-containing dilution fluids may carry over large amounts of non-virus compounds (proteins and phospholipids) which would greatly alter at least the quantitative aspects of the interaction of surface active agent and virus. In

<sup>22</sup> Compare, however, the recent revelation of a high degree of surface activity for the sodium salt of penicillin (77).

addition, most studies have been planned solely on the basis of the demonstration of ability or inability of the surface active agent to inactivate virus activity.

Studies of the purified plant viruses of tobacco mosaic, tomato bushy stunt, and potato "X" in the presence of the anionic compound, dodecyl sulfate, have demonstrated virus inactivation with a concomitant splitting of the nucleic acid from the protein (13, 14, 16, 215). These viruses varied in susceptibility to the action of the dodecyl sulfate, with potato "X" being most susceptible and tomato bushy stunt least susceptible. Within a rather narrow range of experimental conditions (pH, temperature, and dodecyl sulfate concentration), inactivation of tobacco mosaic virus without loss of serological activity can be demonstrated (16) and under these conditions anisotropy of flow is retained. With increased concentrations of dodecyl sulfate, however, more severe molecular changes are produced leading not only to loss of infectivity, but to abolition of serological activity and anisotropy of flow as well. Similar results are possible with the tomato bushy stunt virus although, in general, it is easier to demonstrate destruction of infectivity without loss of serological activity with the tomato bushy stunt virus than with the tobacco mosaic virus. Many aspects of the interaction of dodecyl sulfate and these plant viruses are similar to the effects of urea, as a denaturant, upon the same viruses (15).

In the interaction of cationic compounds with tobacco mosaic virus, aggregation paralleling inactivation has been demonstrated (172). The efficiency of cationic compounds in causing virus aggregation was proportional to the length of their alkyl chain, and concentrations necessary for precipitation of the virus were similar to those necessary for precipitation of such simpler proteins as egg albumin and lactalbumin.

Paralleling the investigations of inactivation of toxins by soap and bile salts, and the attempted use of these inactivated products as vaccines, many investigators have studied the effect of these same compounds on animal viruses with similar ends in view (20, 32, 79, 139, 177). Perhaps the investigations of Klein *et al.* (106) bring out most of the relevant points, other than immunogenic capacity, evident from interactions of animal viruses and surface active agents reported, thus far, in the literature. When anionic, cationic, and non-ionic surface active agents were allowed to interact with animal viruses and bacteriophages, the ability of a surface active compound of a given homologous series to inactivate was found to be proportional to alkyl chain length; a non-ionic surface active compound had no inactivating effect; and various viruses and phages differed from each other in susceptibility to inactivation. One of them, the gamma phage active against *Escherichia coli* B was highly resistant to all surface active agents used. Indeed, on the basis of these results, cationic surface active compounds have been recommended for the isolation of coli phage from sewage (97).

This difference in susceptibility of various viruses and phages to inactivation by surface active agents and the seeming inability of inactivation of some of them has been observed elsewhere (29, 214). Thus, Burnet and Lush (29) utilizing sodium desoxycholate, saponin, and sodium dodecyl sulfate as surface

active compounds found the following order of resistance to inactivation. The list is given in order of increasing resistance: herpes, louping ill, influenza, Sabin's B virus, pseudorabies, myxomatosis, fowl-pox, vaccinia, ectromelia, psittacosis. Psittacosis virus was found to be practically unaffected by the highest concentration of inactivating agents used. Three dysentery phages, two *Salmonella* phages, and two phages active against *Staphylococcus aureus* also showed no inactivation by dodecyl sulfate.

Although high dilutions of surface active agents may fail completely to inactivate the influenza virus (116) and on this basis have been recommended for freeing throat washings, egg fluid, or ground mouse lung from adventitious bacteria (115), many reports of the inactivation of influenza virus by higher concentrations of both anionic and cationic surface active agents exist (29, 108, 110, 214, 222, 223). In the case of the influenza and also the lymphocytic choriomeningitis virus, attempts to recover active virus by removal of the surface active agent were unsuccessful (224). The PR 8 strain of influenza virus, type A, rendered non-infectious by treatment with sodium oleate has been found to retain its immunogenic capacity unaltered (222, 223). Although the antigenic capacity of only this one strain was tested after inactivation by sodium oleate, various other strains of both type A and B influenza virus were shown to be inactivated by the same procedures. Inactivation of virus by surface active agents with retention of antigenic capacity has also been reported with the neurotropic strain of the yellow fever virus (52), but, on the other hand, immunization experiments with lymphocytic choriomeningitis virus inactivated with surface active agents were unsuccessful (224). Although there are early reports of the antigenic capacity of sodium ricinoleate inactivated poliomyelitis virus (112, 143), later work has not confirmed this (113, 162).

#### APPLICATION OF SURFACE ACTIVE AGENTS TO PROBLEMS OF SANITATION

The surface active agents of primary interest in application to problems of sanitation are the quaternary ammonium compounds which, we have seen, have outstanding bactericidal properties. A more concrete idea of the extent of current use of these compounds may be obtained from the latest government statistics (229) showing 1945 production in the United States to exceed 3 million pounds.

Quaternary ammonium compounds have several advantages for consideration as bactericides (191). At effective concentrations they are virtually odorless, tasteless, have low oral toxicity, and are relatively non-irritating to the skin. By virtue of their surface activity they penetrate and wet surfaces. As chemical compounds they are stable, non-corrosive, and neutral. The lack of taste and odor makes them especially desirable in the sanitation of food establishments.

In contrast to these advantages, it is well to keep in mind that the bactericidal efficiency of quaternary ammonium compounds is more markedly diminished by decrease in temperature or presence of organic matter than is true for phenolic compounds. Quaternary surface active compounds are cationic and as such have certain incompatibilities, among which might be mentioned soap

and anionic surface active compounds in general, sodium hexametaphosphate, sodium metasilicate, and acidic dyes. It is especially important to keep these incompatibilities in mind when compounding formulations designed to have high detergent as well as bactericidal properties. Since the bactericidal efficiency of most quaternary compounds is not constant over a wide pH range it is important also to evaluate the compounds at the pH actually to be encountered in particular sanitary applications.

Because of the considerable price differential between the quaternary ammonium and phenolic compounds, accurate evaluation of their relative bactericidal efficiency is most important in arriving at a decision as to their comparative merits in any practical situation. Unfortunately, as we have seen in our discussion of methods of evaluation of these compounds as bactericides, no single test method gives an unequivocal answer to this problem. Indeed, it has been concluded (199) that the only reliable basis for selection of a bactericide lies in actual determination of its utility under the conditions of ultimate use.

Some of the current sanitary applications of quaternary ammonium compounds will be briefly reviewed to give an idea of the scope of their application. In eating establishments they have been recommended for sanitizing dishes, glasses, and utensils (114, 145, 147). Detergency of these bactericidal dishwashing formulations may be increased by the addition of appropriate non-ionic surface active compounds. It should be kept in mind that if the alkalinity of the detergent is sufficiently high to saponify fat the resulting soap will inactivate the cationic quaternary ammonium compound with resultant loss in bactericidal activity (70). The dairy industry finds quaternaries useful in the sanitation of milk cans, dairy machinery (72, 156, 157), cow udders, and milkers' hands (87, 204). In food processing industries these compounds, besides being useful in general environmental and machinery sanitation, have found use in washing dirty eggs, resulting in a reduction of the bacteria count of dried egg pulp (170, 171). An interesting attempt to make fish-packing ice bactericidal by addition of quaternary ammonium compounds has not, as yet, proved successful (225). Many medical uses have been suggested for these compounds. Antisepsis of the skin of the patient and hands of the operator (71, 78, 152) and surgical instruments (88) have been reported. This antisepsis of the skin may be more apparent than real since cationic surface active compounds applied to the hands have been shown to deposit a non-perceptible film which retains viable bacteria underneath it (152). This film has a low bactericidal power on the inner surface whereas the outer surface is strongly bactericidal. Recent interest in combatting air-borne infections is reflected in the use of quaternary ammonium compounds impregnated into blankets or other cotton or woolen textiles (202, 228) to impart an efficient bactericidal activity. The possible use of quaternary ammonium compounds as components of aerosol bomb type bactericides has also been suggested (109, 230).

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## MUCIN AS A RESISTANCE-LOWERING SUBSTANCE

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Mucin preparations of different origins are able to lower the minimum number of microorganisms of low pathogenicity that can cause a fatal infection in an otherwise resistant animal, when the mucin is introduced together with such microorganisms. In this respect their action is similar to that of specific "agresins", and some authors have designated mucin as an "aggressin-like substance" (19).

The importance of the search for resistance-lowering substances lies in the fact that experimental work on many infectious diseases has been handicapped in the past by lack of susceptible small laboratory animals. In certain cases, several millions of microorganisms were found to be required to produce a fatal abdominal infection in a laboratory animal. When so massive a dose is injected, a fatal outcome may be interpreted as due merely to the action of endotoxins liberated in the body through disintegration of the injected bacteria.

A similar difficulty has arisen in attempts to produce experimental infections of the respiratory tract. Thus, the white rat, although highly susceptible to the subcutaneous injection of *Diplococcus pneumoniae* exhibited the symptoms of lobar pneumonia after intratracheal introduction of this organism only when mucin was simultaneously administered (57, 58).

Mucin and some carbohydrates, therefore, have been employed for the following purposes:

A. Production of peritoneal infections and general septicemia with minimal quantities of endotoxin-containing microorganisms in order to exclude the initial endotoxin effect. B. Production of lobar pneumonia by microorganisms in otherwise resistant animals.

Mucin has found a wide application in virulence tests, titrations of the protective effect of immune sera, active vaccination experiments, and tests on the protective and curative effects of chemotherapeutic agents, antibiotics, and bacteriophages. Much study has been devoted to the elucidation of the nature of its effect, its relationship to humoral and cellular defense mechanisms, and its possible influence on the microorganisms themselves. Its chemical and physical properties have been discussed by a number of workers and attempts have been made to use mucin diagnostically to reveal the presence of exceedingly small numbers of microorganisms (84).

### PRODUCTION OF PERITONEAL INFECTIONS WITH THE AID OF MUCIN

1. *Species of microorganisms found to be susceptible to the action of mucin.* It is clear from the aforesaid that the effect of mucin has hitherto been studied mainly on organisms with low virulence for the usual laboratory animals. At least several millions of such microorganisms were required to produce fatal infections. Since doses of such a size contained considerable quantities of



endotoxin, it was difficult to conclude with certainty that a fatal outcome in these cases was due to the virulence and invasiveness of the injected bacteria. Employment of mucin in work on different organisms was therefore dictated primarily by the need for an infection technique which would produce a response

TABLE 1

*Microorganisms whose pathogenicity, if they were injected intra-abdominally, was enhanced by mucin and some other carbohydrates*

FAMILY	SPECIES	EXPERIMENTS REPORTED IN YEARS	REFERENCES
<i>Pseudomonadaceae</i>	<i>Vibrio comma</i>	1942-44	26-28, 74
	<i>Pseudomonas aeruginosa</i>	1939	16
<i>Micrococcaceae</i>	<i>Staphylococcus aureus</i>	1932-45	18, 19, 61, 80, 89
<i>Neisseriaceae</i>	<i>Neisseria catarrhalis</i>	1935	73
	<i>Neisseria gonorrhoeae</i>	1933-43	13, 35, 42, 48, 50, 80
	<i>Neisseria intracellularis</i>	1933-46	1, 3, 7, 11, 12, 23, 39, 40, 46-53, 70, 71, 73, 80, 87
<i>Parvobacteriaceae</i>	<i>Hemophilus influenzae</i>	1937	24
	<i>Hemophilus pertussis</i>	1938	79
<i>Lactobacteriaceae</i>	<i>Streptococcus pyogenes</i>	1932-43	61, 80
	<i>Diplococcus pneumoniae</i>	1932-43	9, 61, 80
<i>Enterobacteriaceae</i>	<i>Escherichia coli</i>	1924-43	2, 16, 41, 80, 82, 83
	<i>Klebsiella pneumoniae</i>	1936	59
	<i>Proteus vulgaris</i>	1939-43	16, 80
	<i>Salmonella typhimurium</i>	1937-43	8, 80
	<i>Eberthella typhosa</i>	1935-47	4, 6, 8, 22, 32, 33, 43-45, 66, 68, 69, 72, 80, 90
	<i>Shigella dysenteriae</i>	1938-47	5, 21, 62, 64-67, 78, 80, 81
<i>Mycobacteriaceae</i>	<i>Shigella paradysenteriae</i>	1940-46	17, 75, 78, 80, 86
	<i>Mycobacterium tuberculosis</i>	1944	53
<i>Viruses</i>	<i>Corynebacterium diphtheriae</i>	1945	20
	<i>Poliomyelitis</i>	1933, 1936	56, 88
	Shope fibroma, Vaccinia virus	1942	10

more closely resembling natural infection than the reaction which follows the simultaneous inoculation of immense masses of highly endotoxic organisms.

As is shown in tables 1 and 2, many have succeeded in lowering the fatal dose of an infecting organism with the aid of mucin. In certain cases they succeeded only in accelerating the death of the experimental animal, as, for example, in the case of *Streptococcus pyogenes* (61) and the S form of *D. pneumoniae* (80). On

TABLE 2

*The minimal lethal doses of different microorganisms inoculated into mice intra-abdominally along with mucin*

MICROORGANISMS	MLD EXPRESSED BY		REFER- ENCE
	Quantity of broth culture	No. of bacteria	
	ml		
<i>Corynebacterium diphtheriae</i>	$1.0 \times 10^{-3}$ (99/106) <sup>a</sup> $1.0 \times 10^{-4}$ (30/32)		20
<i>Diplococcus pneumoniae</i> (R strain)		$10^6$ (4/8)	80
<i>Escherichia coli</i>	$0.5 \times 10^{-4}$ (8/10 to 10/10) $1.0 \times 10^{-9}$ (2/3)		16 80
<i>Eberthella typhosa</i>	$1.0 \times 10^{-5}$ (2/4)	$5 \times 10^2$ (8/10) $5 \times 10^3$ (10/10) 50 (4/10)	32 80 8
<i>Hemophilus influenzae</i>	$0.25 \times 10^{-4}$ (3/4)		24
<i>Hemophilus pertussis</i>	$1.0 \times 10^{-1}$ (225/260) <sup>b</sup>		79
<i>Pseudomonas aeruginosa</i>	$0.5 \times 10^{-1}$ (4/10 to 10/10)		16
<i>Proteus vulgaris</i>	$0.5 \times 10^{-2}$ (9/10 to 10/10) $1.0 \times 10^{-5}$ (4/4)		16 80
<i>Staphylococcus aureus</i>		10 (7/12) $3-12 \times 10$ (7/14) $2.5-12 \times 10^2$ (14/23) $2-11 \times 10^3$ (32/40) $4-6 \times 10^4$ (188/191)	18
	$1.0 \times 10^{-1}$ (7/12) $1.0 \times 10^{-3}$ (3/3)		56 80
<i>Shigella dysenteriae</i> R strain S strain	$0.5 \times 10^{-4}$ (1/3) $0.5 \times 10^{-5}$ (2/3)		5
<i>Shigella paradysenteriae</i>	$0.5 \times 10^{-5}$ (39/48)		17
<i>Salmonella typhimurium</i>		50 (10/10)	8
<i>Vibrio cholerae</i>		$5 \times 10^5$ (80/100)	74

<sup>a</sup> The numerator shows the number of dead animals; the denominator, the number of animals used in the experiment.

<sup>b</sup> Density of undiluted saline suspension 0.7, determined by Gates turbidometer.

the other hand, treatment with mucin lowered the fatal dose of the R form of *D. pneumoniae* from more than  $10^9$  to  $10^6$  organisms (80). In other cases, such as, for example, *Myobacterium tuberculosis*, only larger local lesions were observed (54).

As is shown in table 3, considerable differences exist among the susceptibilities of different strains of the same species towards the action of mucin. These varying behaviors led to extensive studies on the relationship of mucin to the virulence of the variants and types (e.g., R and S form of *Shigella dysenteriae* (5), and different types of *Neisseria intracellularis* (7, 12, 49, 73).

TABLE 3  
*Minimal fatal doses of different Neisseriae inoculated intra-abdominally  
into mice along with mucin*

SPECIES AND TYPES	NO. OF STRAINS TESTED	EXPRESSED BY NUMBER OF BACTERIA	REFERENCE
<i>Neisseria catarrhalis</i>	1	$2 \times 10^6$	73
<i>Neisseria gonorrhoeae</i>		$5 \times 10^6$	13
		$10^8$ (3/3) <sup>a</sup>	80
<i>Neisseria intracellularis</i>			
Type I-III	4	$2 \times 10^3$ (2/2)	73
	3	$2 \times 10^4$ (2/2)	
II	8	$2 \times 10^5$ (2/2)	
VII	1	$2 \times 10^7$ (2/2)	
II	1	10 (1/2)	12
		$10^3$ (2/2)	
	1	10 (2/3)	
		$10^2$ (3/3)	
I-III	8	$10^2$ - $10^3$	58
	5	10	
	1	less than 10	
I-III and II		2 (50% mortality)	7

<sup>a</sup> The numerator shows the number of dead animals; the denominator, the number of animals used in the experiment.

Few attempts have been made to employ mucin in connection with intraperitoneal infection with viruses. Nungester (56), working with poliomyelitis virus in mice, observed a higher mortality in the mucin-treated group than in the saline-treated group, while the results of the serial passages were irregular. In only one out of seven series was inflammation of the spinal cord demonstrated in the fourth generation by histological examination. The experiments of Toomey and Phelps (88) with poliomyelitis virus yielded a 40 per cent mortality in the mucin-treated group as against a 3 per cent mortality in the saline-treated group, but their results are not conclusive, since normal cord with mucin produced a

13 per cent mortality. On the other hand, Clemmesen and Krag Andersen (10) observed that the spread of fibroma virus following intra-abdominal inoculation was favored by mucin. Mucin also increases the local reaction to the intradermal injection of fibroma and vaccinia viruses, and reduces the minimal effective dose.

2. *Differences in the effectiveness of different mucin preparations.* It may be noted that different mucins possess varying degrees of activity. Nungester *et al.* (61) used a dried gastric mucin which had been rendered innocuous to mice either by heating at 140 C for 1 hour or by exposure to 80 per cent ethyl alcohol for one week. Commercial gastric mucins from different sources have been compared (59, 66, 80). Different lots contained peptone (59) and inorganic substances (66) in varying amounts. In his first experiments, Miller (48), used commercial hog mucin. Later, a special preparation ("Granular mucin", Wilson Laboratories, Chicago) was introduced (51, 52) and gained wide application. Tunnicliff observed no differences in activity between salivary and gastric mucin (89). Marked differences between two mucin preparations were, however, found by Olitzki and Koch (66). The more effective commercial preparation contained a large amount of ash (40.5 per cent), consisting mainly of aluminum. It is possible that the varying degrees of activity of mucins are associated with the presence of blood group A-substance which is able to form complexes with the conjugated proteins of bacteria. Morgan and King observed that group A-substance derived from hog gastric mucin acts as a resistance-lowering factor (55).

3. *The influence of the method of preparation of the mucin suspension on its effectiveness.* Fothergill *et al.* (24) in experiments with *H. influenzae* found differences in effectiveness between different mucin suspensions prepared according to the methods of Nungester or of Miller. According to Nungester's method (59) the solutions are brought to a viscosity of 10, whereas according to Miller's method (48, 51, 52) a constant concentration (5 per cent) is maintained independently of the viscosity of the preparation. McLeod (46) examined different mucin solutions prepared from an identical source. Four types of solution were prepared: 6 per cent "clear mucin" with a viscosity of 2 (prepared according to the original method of Miller (48) by sterilizing at pH 7.3), and three solutions of "whole mucin" (autoclaved at acid pH according to Miller's (51, 52) later procedure). For concentrations of 1, 4.5, and 10 per cent of "whole mucin" (showing viscosities 1, 2, and 10, respectively), minimal fatal doses with  $10^{-3}$ ,  $10^{-6}$  and  $10^{-8}$  ml broth culture of *N. intracellularis* were observed. In a comparison of 4.5 per cent "whole mucin" and 6 per cent "clear mucin" suspensions, both of viscosity 2, the average fatal dose with the "whole mucin" was found to be  $10^{-5}$  ml of broth culture, whereas ten times this dose was required to produce infection when "clear mucin" was employed.

4. *The resistance-lowering action of different carbohydrates compared with that of mucin.* In studies parallel to those on the various mucin preparations, carbohydrates of different origins and of varying degrees of activity were tested for the same purpose. Many observations have been made of their physical and

TABLE 4

*Carbohydrates of different origin used as resistance lowering substances  
in intra-abdominal infections of experimental animals*

SUBSTANCES EXAMINED	EFFECTIVE CONCENTRATION	MICROORGANISMS TESTED	REFERENCE
	%		
Animal products:			
Glycogen	5.0-10.0	<i>Eberthella typhosa</i>	68
Blood group A antigen (from mucin)	—	—	55
Plant products:			
Agar	0.3-10.0	<i>Neisseria intracellularis</i> <i>Eberthella typhosa</i>	1, 61 66, 68
Gum acacia	2.0-10.0	<i>Neisseria intracellularis</i> <i>Eberthella typhosa</i> <i>Diplococcus pneumoniae</i>	1, 59 68 9
Gum tragacanth	1.5- 2.5	<i>Neisseria intracellularis</i> <i>Escherichia coli</i>	46 2, 82, 83
Mannan (carob)	5.0	<i>Eberthella typhosa</i>	64, 68
Inulin	5.0-10.0	<i>Eberthella typhosa</i>	68
Cellulose (cotton)	5.0	<i>Eberthella typhosa</i>	68
Bacterial products:			
Levan ( <i>Aerobacter levani-</i> <i>cum</i> )	0.3-2.0	<i>Eberthella typhosa</i>	68
Levan ( <i>Bacillus subtilis</i> )	1.0-2.0	<i>Eberthella typhosa</i>	68
Dextran ( <i>Leuconostoc mes-</i> <i>enteroides</i> )	1.0-2.0	<i>Eberthella typhosa</i>	68
Cellulose ( <i>Acetobacter xyli-</i> <i>num</i> )	2.0	<i>Eberthella typhosa</i>	68
Carbohydrates of:			
<i>Corynebacterium diph-</i> <i>theriae</i>	5.0	<i>Neisseria intracellularis</i>	1
<i>Escherichia coli</i>	0.2-2.0	<i>Eberthella typhosa</i>	64
<i>Eberthella typhosa</i>	0.1-2.0	<i>Eberthella typhosa</i>	64
<i>Salmonella enteritidis</i>	0.2-2.0	<i>Eberthella typhosa</i>	64
<i>Klebsiella capsulata</i>	0.3-2.0	<i>Eberthella typhosa</i>	64

chemical properties and their abilities to lower the resistance of the experimental animals in comparison with the properties and the effect of mucin, and a brief list of these substances is given.

Table 4, which lists these substances according to source, shows that higher

concentrations of carbohydrates of plant or animal origin are generally required to lower resistance than of bacterial carbohydrates. The only known exception, agar, lowers the resistance of mice to *Eberthella typhosa* even when employed in a 0.3 per cent concentration (66).

In this respect the range of effectiveness of mucin is similar to that of bacterial carbohydrates. One-tenth to 1.0 per cent concentrations of a certain mucin preparation were sufficient to lower the minimal lethal dose of *E. typhosa* (64), and no animal or plant product has yet been found to equal mucin or bacterial polysaccharides in this resistance-lowering effect. Among the plant products starch and dextrin derived from it, galactomannan, pentosan, and pectin were found to be inactive in 10 per cent concentrations. Mono-, di- and trisaccharides were found to be ineffective (68).

5. *Combined administration of mucin together with other substances.* Nungester *et al.* (61) attempted to augment the effect of mucin by means of the previous injection of India ink, in order to block the fixed phagocytic cells. The procedure failed to increase the pathogenicity of staphylococci or streptococci in mice.

Miller and Castles (52), and McLeod (46) observed a prolonged persistence of "granular material" in the abdominal cavity of mice inoculated with mucin. Whole mucin which contained fine white granules was more effective than granule-free clear mucin of the same relative viscosity. Olitzki and Koch (66) found that a mucin preparation with a high content of insoluble granules is more effective than a preparation consisting of a homogeneous, opaque solution. Since the more effective, granular preparation contained much more inorganic material, notably aluminum, the effect of absorbents which *per se* exhibit only slight pathogenizing activity was next studied. It was shown that the pathogenizing activity of an agar gel and of clear mucin on *Shigella dysenteriae* and *Escherichia coli* can be enhanced by the inclusion of talc, fuller's earth, norit A, or kaolin in the mixture. Similar results were obtained in work on levan, mucin, gelatin, and certain inactive carbohydrate fractions of mucin (64, 66). Experiments with *E. typhosa* (68) showed that kaolin exerts a marked effect on the resistance-lowering activity of some carbohydrates (cellulose preparations, gum acacia, glycogen, mannan, inulin). The mixing of two viscous substances sometimes produced an augmentation of the activity of the more active constituent. According to McLeod (46) the lethal dose of a meningococcus culture (defined as the dose which caused the death of 7 out of 10 mice) varied as follows:  $10^{-3}$  ml in 1.5 per cent gum tragacanth (viscosity 10);  $10^{-3}$  ml in 1 per cent whole mucin (viscosity 1); and  $10^{-7}$  ml in a mixture of 1 per cent whole mucin and 1.5 per cent gum tragacanth (viscosity 10).

6. *Properties of mucin compared with those of other carbohydrates employed in intraperitoneal infections.* *Viscosity.* Attention was first drawn to viscosity as an important property of mucin. Many authors have supposed that high viscosity is an essential characteristic of a resistance-lowering substance. Nevertheless, even the early experiments failed to lend consistent support to this view. Nungester *et al.* (59) found that gum acacia and mucin solutions of relative viscosity 10 are more effective as resistance-lowering substances than are solu-

tions with viscosities 2 and 20. Similar results were obtained by Anderson and Oag (1). The sample of mucin with highest activity dissolved in saline almost completely; in 5 per cent concentration this sample showed only low viscosity. A similar resistance-lowering potency was also exhibited by a highly viscous 10 per cent solution of agar, but a viscous gum acacia solution was inactive. McLeod (46), in her re-examination of the problem of the significance of viscosity, showed by comparative experiments with "whole mucin", clear mucin, and gum tragacanth that pathogenizing activity and viscosity are not correlated.

Olitzki *et al.* (68) compared pathogenizing activity and viscosity in a series of carbohydrates. The highly viscous group (viscosity 10 or more) comprised agar, dextran, and galactomannan. Of this group only agar and dextran showed pathogenizing activity. The group of substances with a relative viscosity of about 2 included glycogen (10 per cent) and levan (2 per cent). The former was almost devoid of pathogenizing activity, whereas the latter was highly effective. The group of inactive polysaccharides included several substances of high viscosity (galactomannan, mannan, starch). It is clear, therefore, that viscosity is not an absolute determinant of resistance-lowering activity, and that it does not, *per se*, confer this activity on an injected substance.

*Chemical constitution.* Consideration has been given to the question whether the pathogenizing activity of mucin preparations is due to protein or carbohydrate. Anderson and Oag (1) found that a protein fraction of mucin is highly active and that the carbohydrate fraction is inactive. In view of the known activity of agar and diphtheria polysaccharides, this finding was somewhat surprising, but was in keeping with negative results which had been obtained with some other polysaccharides such as starch, inulin, and dextrin. On the other hand, Morgan and King (55) observed that the polysaccharide amino acid complex isolated from hog gastric mucin acted as a resistance-lowering substance. Olitzki *et al.* (68) re-examined this question in tests involving a larger series of polysaccharides. It was found that pure polysaccharides are able to lower the resistance. The monosaccharide from which the polysaccharide is built up is not, in itself, the pathogenizing agent. Different polysaccharides built from the same sugar showed markedly different pathogenizing activities. The findings were consistent with the view that the sugar unit of which a polysaccharide is composed does not play an essential rôle in determining whether the material is an active one. The resistance-lowering carbohydrates included both  $\alpha$  and  $\beta$  linkage types, furanoside as well as pyranosidic units, and intersaccharidic C-C linkages of the 1-4 (cellulose, glycogen), 1-6 (dextran), 2-1 (inulin), and 2-6 (levan) types. It was suggested, therefore, that the resistance-lowering activity of a polysaccharide is a function of patterns which are characteristic of the colloidal state. The activity depends only to minor degree on the chemical structure, configuration and manner of linkage of the individual repeating unit, or on the presence of polar groupings in the carbohydrate polymer. The view that the carbohydrate as well as the protein fraction of mucin may lower the host resistance is supported by an observation by Olitzki and Koch on fractions derived from two different mucin preparations: the protein as well as the carbo-

hydrate fraction were able to lower the resistance of mice (66). Similar results were obtained in recent experiments with different fractions derived from the somatic antigen of *S. dysenteriae*. The undegraded carbohydrate as well as the conjugated protein were found to be able to lower the resistance of mice to *S. dysenteriae* and *E. typhosa* (64). It is obvious that the host animal may be able to decompose and eliminate certain polysaccharides more readily than others. The resistant types of molecules may therefore have a more extended influence. This consideration led to experiments which are summarized in the following paragraph.

*Rapidity of absorption of mucin from the abdominal cavity.* Many authors have reported experiments dealing with the dependence of the resistance-lowering effect on the lapse of time between the injection of the mucin and the inoculation of the bacteria. Nungester *et al.* (59) found 10 per cent mucin lowered resistance 12 hours after injection, and was still slightly active even 16 hours after injection. According to Miller and Castles (52) the pathogenizing ability of a 5 per cent mucin suspension decreases within three to five hours after its injection. McLeod (46) concluded that the effectiveness of different preparations of mucin is inversely proportional to the rapidity with which they are absorbed and removed from the abdominal cavity. Olitzki and Koch (66) found a highly active mucin still effective six hours after the injection, whereas another mucin preparation of low activity was inactive one hour after its inoculation. Agar was inactive after two hours, but administered together with kaolin its activity lasted for seven hours. It was concluded that the addition of an adsorbent (like kaolin) retards the absorption of the agar from the abdominal cavity. These observations were confirmed by the experiments described in the following paragraph.

7. *Quantitative studies on mucin employed for intraperitoneal infections. Effect of concentration of mucin in the inoculum.* Nungester *et al.* (59) compared the effects of varying concentrations of mucin suspensions. The relationship between the concentration and the pathogenizing effect was not linear. A 20 per cent autoclaved suspension, for instance, had the same effect as a 4 per cent alcohol-sterilized suspension of the same viscosity. McLeod (46) found that the concentration of the mucin suspension influences the response. In 1, 4.5, and 10 per cent mucin, the minimal lethal dose of a meningococcus culture was  $10^{-2}$ ,  $10^{-6}$  and  $10^{-8}$  ml, respectively. It could be expected that the effect of the more highly concentrated mucin would persist for a longer time. When the bacteria were inoculated 12 hours after the mucin injection, the fatal dose was  $10^{-1}$  ml in the animals which had received 4.5 per cent mucin and  $10^{-8}$  ml in those which had received 10 per cent mucin.

*The volume of inoculum.* It is clear that in low concentration mucin is resorbed more slowly than in high concentration. The effect is more marked if the volume of the administered solution is increased without parallel change in the concentration. Nungester *et al.* (59) found for a mucin solution of viscosity 10 that the minimal effective dose in mice is 0.2 ml. In rats the lowest effective dose is about 2.0 ml, or 10 times that in the mouse. The results show that mucin after inoculation may sometimes be diluted by the formed exudate to a point at



which it is no longer active. McLeod (46) compared the effect of different quantities of 4.5 per cent mucin solution on the pathogenicity of meningococci. With 1.0, 0.5, and 0.25 ml of mucin solution the respective minimal lethal doses of meningococci were found to be 83, 8300 and 83,000.

8. *Mechanism of action of mucin in intraperitoneal infections. Change in virulence of bacteria induced by growing them in vitro in the presence of mucin.* Nungester *et al.* (59) concluded from experiments with streptococci and with *Bacillus anthracis* that cultivation of bacteria in the presence of mucin *in vitro* does not appreciably increase their virulence. Attempts by Rake (72) and by Sindbjerg-Hansen (80) to enhance the virulence of *E. typhosa*, *E. coli* and *N. intracellularis* by cultivation in the presence of mucin were not successful.

*Change in virulence of bacteria in vivo in the presence of mucin.* Nungester *et al.* (59) concluded from experiments with *B. anthracis* that mucin is a pathogenizing substance because it helps bacteria to survive in the animal body, and that, in addition, it increases the virulence of the surviving bacteria. Miller and Castles (52) working with meningococci and Sindbjerg-Hansen (80) working with meningococci and *E. coli* arrived at opposite results. Organisms recovered from exudate or blood of moribund mice proved no more virulent than cultures grown on artificial medium. Fothergill *et al.* (24) observed that after 10 passages through mice together with mucin, the ability of *H. influenzae* to kill mice in the absence of mucin was not increased. Observations with other microorganisms were not made.

*Mucin as a culture medium.* Miller and Castles (52) originally thought that mucin exerts its effect by serving as a culture medium in the body of the mouse. This explanation seemed reasonable at the time, in view of the finding that meningococci were able to grow *in vitro* in the particular batch of mucin then employed. It was subsequently observed, however, that other mucins had different effects in this respect; the preparation which was most effective in promoting infection entirely failed to support growth *in vitro* (52). Keefer and Spink (35) carried out experiments with gonococci and Sindbjerg-Hansen (80) with meningococci inoculated into mucin or mucin-broth mixtures. Their experiments showed that mucin, alone, is a poor culture medium for continuous growth of neisseriae. Attempts by McLeod (46) to maintain continuous growth of meningococci in either mucin or in peritoneal fluid alone were unsuccessful. But when one part of peritoneal fluid was added to two parts of mucin, growth occurred with small inocula of meningococci, and subculture from this mixture was obtained. McLeod, therefore, concluded that mucin which is enriched by peritoneal fluid is an excellent medium for meningococci.

*Lethal effects of mucin.* In early experiments with mucin (59), it was reported that mucin alone does not kill mice if injected into the peritoneal cavity. This result has often been confirmed since tests of the pathogenicity of bacteria in mucin have frequently been paralleled by control tests on the effect of mucin without added bacteria. Mucin and certain carbohydrates are themselves non-toxic, but exert a lethal effect when their resorption is delayed by the addition of kaolin. An effect of this kind was observed by Olitzki *et al.* (68) in the use of 1

to 2 per cent levan solutions obtained from *Aerobacter levanicum* and 5 to 10 per cent mucin solutions.

*Anticomplementary action.* Keefer and Spink (35) observed no anticomplementary action when they added 0.5 ml of defibrinated human blood to 0.1 ml of a 5 per cent suspension of mucin. Olitzki found that kaolin, which enhances the action of various resistance-lowering substances, strongly absorbs all four components of complement (middle and end piece, third and fourth component). Low concentrations of this absorbent, (0.1 to 0.5 per cent added to guinea-pig complement diluted 1:20) reduced the complement titer (63). In recent experiments with complement diluted 1:20, it was demonstrated that mucin and agar lower the titer of the complement and that this action is enhanced by small quantities of kaolin (64). The question is complicated by the fact that the animal usually employed in experiments with mucin is the mouse. As has been reported by many authors (34), mouse blood does not contain the whole hemolytic complement. The end piece is not present but can be added in the form of guinea-pig end piece. In my own experiments neither the blood nor the peritoneal fluid of the mouse could be used as hemolytic complement; and it seems, therefore, that bacteriolysis in the peritoneal fluid of this animal takes place by a mechanism which does not require the whole complement (64).

*Acquisition of a protective covering.* Tunnicliff (89) failed to demonstrate an artificial capsule when staphylococci and streptococci suspended in mucin were dried and stained by means of capsular stains. When pneumococci and lactobacilli were mixed with gastric mucin it was found that the organisms failed to take up vital stain. In experiments *in vivo*, it was demonstrated that only stained cocci which are devoid of mucin coating are taken up by polymorphonuclear leucocytes. A coating effect was recently demonstrated by agglutination in Shiga antiserum of typhoid bacteria having the undegraded polysaccharide of *S. dysenteriae* adsorbed on their surface (64). It seems that this coating effect of mucin and other carbohydrates together with their ability to persist for a sufficient time in the abdominal cavity are the chief factors determining the other activities described below, such as the ability to prevent phagocytosis, intracellular digestion, and antibody-uptake.

*Inhibition of phagocytosis by mobile phagocytic cells.* Nungester *et al.* (59) observed the presence of numerous minute particles in mucin. They observed no decrease of phagocytosis when they sought to ascertain whether these particles block the phagocytic cell *in vitro*. Miller and Castles (52) reported that microscopic examination of peritoneal exudates revealed degenerative changes in many of the cells and the ingestion of insoluble particles of mucin especially by polymorphonuclears. Some of the cells seemed to have been disabled by the mucin, but the infected exudate still contained numerous healthy looking polymorphonuclears. In the peritoneal exudates of mice protected by immune serum, the cells had a similar appearance, except that more of them contained meningococci. The effect on the mobile phagocytes must, therefore, represent only a part of the action of mucin. McLeod (47) failed to find evidence that clear mucin interferes with the ingestion of meningococci by phagocytic cells. On

the other hand, granular whole mucin interfered with phagocytosis by inhibiting the circulation of leucocytes and by encumbering the cells with granules. According to McLeod (47) this effect can be of little importance. It has been pointed out by McLeod that whereas it was possible to reduce the lethal dose of virulent strains of meningococci only by one fourth by blocking the phagocytes with carmine particles, a million-fold increase of invasiveness can be obtained with granular mucin.

This view is supported by the observation that mucin is unable to exert an influence when leucocytosis is already present (19, 52, 62, 82). Ercoli *et al.* (19) injected staphylococci together with mucin after local leucocytosis had been induced with aleuronat. In the presence of the leucocytes the destruction of the staphylococci was rapid. It was concluded that mucin does not interfere with the action of leucocytes when they are already present in peritoneal fluid. Steinberg found that the peritoneum of dogs protected if "hyperleucocytic pre-immunity" was produced (82).

Miller and Castles (52) showed that mucin administered intra-abdominally increases the resistance of the animal to meningococci injected 24 to 29 hours later. This result was ascribed to a local stimulation of the defense mechanism, and no evidence of humoral immunity was found. Oerskov (62) in his experiments with *S. dysenteriae* obtained similar results. Tunncliff (89) reported that when staphylococci are injected with mucin it is possible to demonstrate the presence of mucin-coated nonstained cocci as late as three hours after the injection. Only the stained cocci which are noncoated with mucin, are taken up by the polymorphonuclears.

This observation is important because some authors have ascribed the lowered phagocytic activity to a blockade effect produced by the granular material in mucin, while others have considered that mucin damages phagocytes directly. In Tunncliff's work, however, the lowered phagocytic activity is ascribed not to an effect on the cells, but to a direct coating action on the bacteria.

The other mechanisms involved are of minor importance. The blockading of the white cells by granules plays a rôle only when granules are associated with mucin (47, 52); and when granular particles were employed alone (61, 66) their action was not equal to the mucin effect. Furthermore, damage to individual cells by mucin (52, 66) seems to be of minor importance. The increase in resistance which has been observed in the presence of leucocytosis by many authors (19, 52, 62, 82) proves that if the number of the phagocytes is high enough the damage ascribed to mucin becomes relatively insignificant and is inadequate to break down the high resistance which is conferred by hyperleucocytosis.

*Inhibition of the appearance of leucocytes in the abdominal cavity.* Ercoli *et al.* (19) reported that mice given 50,000 staphylococci in saline exhibit a strong leucocytic reaction some hours after the inoculation, and no cocci could be seen microscopically at this time. When the same number of staphylococci was given with mucin a strikingly different picture developed. In this case only isolated leucocytes appeared, and the cocci in the peritoneal fluid survived be-

cause they were not destroyed by the leucocytes which normally would have appeared in large numbers. Similar results were obtained with heated staphylococci, meningococci and *C. diphtheriae*. Other observers (66) noted the adsorption of free phagocytes to kaolin-agar or kaolin-mucin clots in the peritoneal fluid after the injection of kaolin-agar or kaolin-mucin. The number of free polymorphonuclears decreased rapidly after the injection of these substances. Fragments of the cells were observed microscopically in the kaolin-agar clots.

Decrease in the number of leucocytes in the abdominal cavity was also observed by Steinberg (82) in dogs inoculated with *E. coli* suspended in gum tragacanth. Miller and Castles (52) found that cellular elements did not begin to accumulate in the abdominal cavity until three hours after the inoculation of meningococci suspended in mucin or sterile mucin, and did not become abundant until the ninth hour. At first the cells consisted chiefly of small round cells, but the proportion of polymorphonuclears rose during the second three-hour period and reached 66 to 74 per cent. Oerskov (62) reported that the increased migration of phagocytes into the peritoneum in the first hours after inoculation of *S. dysenteriae* is hindered if the organism is administered together with mucin. Sindbjerg-Hansen found the migration of polynuclear leucocytes into the abdominal cavity to be regularly retarded by mucin (80).

In summary it may be stated that the number of the phagocytes in the abdominal cavity decreases during the first hours after the intra-abdominal administration of mucin, but later increases, the delayed increase being associated with an increased resistance of the animal to intraperitoneal infection. The question arises whether this mechanism operates in the cases of all species of microorganisms whose pathogenicity is affected by mucin, since the rapid destruction of certain species of microorganisms in the abdominal cavity (24, 66) is due mainly to the bactericidal action of peritoneal fluid.

*Inhibition of activity of fixed tissue phagocytes and increased permeability of the barrier between the abdominal cavity and the blood stream.* Miller and Castles (52) carried out experiments to ascertain whether fixed tissue phagocytes, which constitute part of the barrier between the peritoneal cavity and the vascular system, are injured by mucin with a resulting increase in the permeability of the barrier to organisms. Mice with recently injected mucin in the peritoneal cavity succumbed to intravenous injections of smaller numbers of meningococci in mucin than did mice which had not received mucin intraperitoneally. Examination of the peritoneal cavity showed that it becomes infected soon after the introduction of the cocci into the circulation. This fact, together with the observation that the meningococci which are injected with mucin into the abdominal cavity soon invade the blood stream suggests that mucin renders the barrier between the peritoneal cavity and the vascular system permeable to the invading organisms in both directions. A similar observation was made by Benians (2) in rabbits with *E. coli* given intravenously.

Almost all authors have emphasized the rapidity with which microorganisms appear in the blood stream after their intraperitoneal inoculation together with mucin. This has been confirmed for many bacteria such as *N. intracellularis*

(52), and *H. influenzae* (24). Olitzki *et al.* (67) found *S. dysenteriae* in the blood stream one hour after their inoculation together with either broth or mucin. When the bacteria in broth were injected, they permanently disappeared from the blood stream after two hours; but when they were injected in mucin, the organisms persisted in the blood. In some of the mice, the bacteria inoculated in the presence of mucin disappeared from the blood after two hours, reappeared again in considerable numbers after four hours, and then increased steadily. Parallel examinations of the peritoneal fluid and of the abdominal organs showed that a local abdominal infection concurrently makes rapid progress. It is reasonable to suppose that the local focus pours a steady stream of invading bacteria into the blood. Similar observations were made by McLeod (47). When large quantities of meningococci (culture diluted  $10^{-1}$ ) suspended in Ringer's solution were given, organisms appeared in the blood stream 0 to 20 minutes after the injection. The numbers soon fell, however, and after six to seven hours the blood was sterile. When a smaller quantity of meningococci (dilution  $10^{-3}$ ) was administered suspended in mucin, the organisms appeared in the blood immediately after the inoculation. The number then fell rapidly during the first hour, but began to increase after 2.5 hours. The progressive increase continued and persisted until death occurred.

From these and similar experiments it may be concluded that the appearance of bacteria in the blood stream cannot be attributed to augmentation of the permeability of the abdominal cavity by mucin. Recent experiments by Olitzki (64) with a series of mucin preparations and bacterial carbohydrates revealed no spreading activity. It can easily be demonstrated that early invasion of the blood stream takes place even in the absence of mucin, but tends in this case towards progressive sterilization. In the presence of mucin, however, the rapid progress of the abdominal infection steadily pours new invaders into the vascular system. After local multiplication of the mucin-coated bacteria in the abdominal cavity has started, the reappearance of the bacteria in the blood stream represents the beginning of a fatal bacteremia. The importance of the second invasion of the blood stream has been demonstrated directly with gonococci (13) and with *E. coli* (80). When the infected mucin emulsion was washed out of the peritoneal cavity of mice with a sterile salt solution two and four hours after injection, the animals recovered although they already had bacteria in the blood stream at the time of this operation. Removal of the abdominal focus of the infection evidently prevented the renewal of the invasion, and the body was thus enabled to overcome the incipient septicemia.

*Inhibition of intraphagocytic digestion.* Nungester *et al.* (59) reported that although mucin does not interfere with phagocytosis, it prevents the *in vitro* destruction of the bacteria in the phagocytic system. McLeod (47) investigated this subject with heat-killed meningococci. In mice receiving the meningococci in Ringer's solution the number of intracellular organisms decreased rapidly and progressively. In mice receiving the organisms coated with mucin there was a decided increase in the number of the intracellular organisms seen after one hour, after which a very gradual decline was noted. At the end of six hours the num-

ber of intracellular organisms in the mucin-treated group exceeded the number seen in the control group, while the extracellular organisms disappeared at the same rate in both groups of mice. It was concluded that the degree of phagocytosis is the same whether the vehicle of the inoculum is Ringer's solution or mucin. It is only the intracellular digestion of organisms which is inhibited by the presence of mucin.

*Inhibition of antibody absorption by microorganisms.* A theory which suggests that mucin acts as a kind of hapten in hindering the absorption of antibody to the infecting microorganism has been investigated by Miller and Castles (52). They found that anti-mucin serum fails to protect mice against infection with meningococci, and that mucin injected subcutaneously fails to immunize against infection. They were unable to detect an antigenic relationship between mucin and meningococci. Similar observations were made by Sindbjerg-Hansen in experiments with *S. paradysenteriae*, *E. coli* and *N. intracellularis* (80). On the other hand, Keefer and Spink (35) found that mucin depresses the bacteriolytic effect of blood on gonococci, the strength of the depressant effect being dependent on the amount of antibody present. These investigators suggested that mucin interferes with the sensitization of the microorganism by antibody. McLeod (47) found that mucin lowers the bactericidal effect of fresh mouse serum on meningococci, and Sindbjerg-Hansen found that in normal human serum it has a similar influence on *E. coli*, *S. dysenteriae* and *N. intracellularis* (80). Oerskov (62) also concluded on the basis of his experiments with *S. dysenteriae* that mucin impedes the normal, extracellular, bactericidal effect of peritoneal fluid, in addition to its influence on phagocytosis.

Olitzki *et al.* (69) studied the inhibiting effect of mucin and other carbohydrates on the agglutination of *E. typhosa* in anti-O serum. Pronounced reduction of the agglutination titer was observed in the presence of mucin, dextran, levan, and pectin. In the presence of these substances the uptake of antibody nitrogen by the bacteria was completely inhibited. But it was impossible to detect a visible precipitin reaction between these substances and the anti-typhoid serum. The question whether mucin acts as a hapten which combines with the antibody, or by means of a coating effect on bacteria, thus blocking the specific receptors on the surface of the microorganism, could definitely be elucidated (64). It was easily demonstrated that the pathogenicity of *E. typhosa* for mice was enhanced not only by its homologous polysaccharide, but to nearly the same extent also by certain mucin preparations and polysaccharides from other microorganisms such as *Shigella dysenteriae*, *Proteus vulgaris*, *Klebsiella capsulata*, *Escherichia coli* and *Salmonella enteritidis* without regard to their antigenic relationship with the polysaccharide of *E. typhosa* (64). Furthermore, it was possible by agglutination tests to demonstrate the coating of the typhoid strain 0901 by the undegraded polysaccharide of *S. dysenteriae*.

9. *The mucin technic in experiments on active and passive immunization.* Many authors have employed the mucin technic in experimental studies on active and passive immunization. When these experiments were carried out with bacterial species which do not produce exotoxins and do not contain labile antigens in

additions to the somatic antigen, the results obtained *in vivo* with the aid of the mucin technic correspond to those obtained with the *in vitro* titration of antibodies. The mucin technic was employed in immunization experiments with *Hemophilus influenzae* (24), *H. pertussis* (79), *Shigella paradysenteriae* (78, 86) and *Vibrio cholerae* (28, 74).

In the case of *Shigella dysenteriae*, the results of the immunization experiments were sometimes obscured by the presence of the neurotoxin in addition to the somatic antigen. In active immunization experiments, somatic antigen (5), vaccines (78), as well as toxoids (21, 65) were employed. In passive immunization experiments, the mucin technic proved to be useful in comparative studies of the effects of antibacterial and antitoxic sera (67, 81). The experiments demonstrated the different effects of both types of antisera on the course of the infection (67), the high effectivity of antibacterial sera against feebly toxigenic S strains, and the high effectivity of antitoxic sera against toxic R strains (81). The mucin technic was introduced in immunological studies on *Eberthella typhosa* by Rake (72). Later, the method was used for active (4, 33) as well as passive (6, 32, 33, 90) immunization studies.

The results showed clearly that where infection is produced by small inocula administered with mucin no difference between O and Vi antigen is revealed. As has been shown by Boivin (6), the effect does not depend on the production of Vi or O antibodies in the serum of the mouse. In view of the possibility thus created of testing the protective action of a vaccine independently of antibody production, experiments measuring the potency of different typhoid vaccines were undertaken. Luippold (45) defined an immunogenic unit (TIU) as that amount which affords protection to all of four mice (16 to 18 g) of a specified breed, which are inoculated in a twice repeated test with  $10^8$  virulent organisms of a strain of *E. typhosa* having a minimal fatal dose of 50 to 500 organisms in 0.5 ml of 5 per cent mucin. Among other problems studied with the aid of the mucin technic, the following deserve mention: the production of cross immunity between members of the typhoid-paratyphoid group, and the immunological relationship between the V forms of *E. typhosa* and *S. ballerup* (43, 44).

The study of the therapeutic effect of antisera on meningococcal infection with the aid of the mucin technic was begun in 1935 (71). Several modifications of the technic were introduced (71, 73, 52), and with the help of these technics different problems in serum production have been examined, e.g., the possible existence of a relationship between the virulence of a strain and the protective activity of its antiserum (11), whether antiserum should be produced by immunization with bacterial filtrates rather than by immunization with whole bacteria (39), whether monovalent antiserum has a higher relative potency than polyvalent antiserum (40), and whether an optimum balance between I-III and II antigens prevails in the accepted methods of preparing polyvalent antisera (40). Serological and immunological characteristics of certain group II strains which differed from other group II cultures have been revealed by the mouse protection test. The findings paralleled those observed in other serological reactions *in vitro* (12). It has been shown that a definite correlation exists

between the type-specific precipitins (12, 70), agglutinins (23), and mouse protection. A mouse protection unit for the standardization of anti-meningococcus serum has been proposed (7).

10. *The use of the mucin technic in experimental work with chemotherapeutic agents, antibiotics, and bacteriophages.* Many authors have employed the mucin method in experimental therapeutic studies. Table 5 presents a brief summary of such studies in which positive results were observed.

TABLE 5

*Studies on chemotherapeutic agents, antibiotics, and bacteriophages in intra-abdominal infections with the aid of mucin*

INFECTING MICROORGANISMS	THERAPEUTIC AGENTS	REFERENCE
<i>Corynebacterium diphtheriae</i>	sulfadiazine, penicillin	20
	sulfa-pyridine, -thiazole, -methylthiazole	80
<i>Eberthella typhosa</i>	sulfanilamide	8
	bacteriophages	22
<i>Escherichia coli</i>	sulfanilamide	16
	sulfa-thiazole, -diazine	41, 80
	sulfa-methylthiazole, -pyridine	80
<i>Neisseria intracellularis</i>	Sulfanilamide, sulfapyridine	3, 23
	sulfa-thiazole, -methylthiazole	80
	penicillin, streptomycin	49, 50, 53
<i>Neisseria gonorrhoeae</i>	diamidinodiphenylsulfon derivatives	42
	sulfanilamide	13
	penicillin, streptomycin	50
<i>Proteus vulgaris</i>	sulfanilamide	16
	sulfa-pyridine, -thiazole, -methylthiazole	80
<i>Pseudomonas aeruginosa</i>	sulfanilamide	16
<i>Shigella paradysenteriae</i>	sulfanilamide, sulfa-thiazole, -pyridine, -methylthiazole	17, 80
<i>Vibrio cholerae</i>	sulfa-thiazole, -pyridine, succinyl-sulfa-thiazole	27

Thomas and Dingle (87) used type I of *N. intracellularis* to study the antagonistic effect of para-aminobenzoic acid against sulfadiazine *in vivo*. One ml of a given number of cocci (100,000 to 100) in 3 per cent mucin was inoculated intra-abdominally into mice. Thirty minutes later 0.005 to 0.0005 mg of sulfadiazine was administered in the same way. If para-aminobenzoic acid was given in eight subcutaneous injections of 0.5 mg each, at intervals of three hours, the protective action of sulfadiazine was lowered by the para-aminobenzoic acid.



*The Production of Lobar Pneumonia with the Aid of Mucin*

The use of mucin in the production of lobar pneumonia (57, 58) was preceded by the employment of starch by Terrel and Robertson (85). The effects of both substances on the development of the inflammation is similar. The effect of starch on the production of pneumonia was studied in dogs by Robertson *et al.* (76, 77); and the mucin effect in rats was studied by Nungester and Jourdonais (57, 58), and by Gunn and Nungester (31). Robertson *et al.* observed that one hour after implantation pneumococci still lie principally within air sacs enveloped by starch, but are already beginning to migrate from the original focus. An outpouring of edema fluid into the air spaces is one of the earliest reactions of the body of the growth of pneumococci in the lungs. Gunn and Nungester (31) showed that edema fluid carries the pneumococci through the smaller air passages and pores of Cohn. The organisms propagate rapidly in this fluid so long as it remains poor in leucocytes. Leucocytic infiltrates appear only during a second stage of the infection. Before infiltration occurs, mucin may affect the activity of the normal eliminatory mechanism which keeps the respiratory tract practically sterile.

Quantitative experiments, as presented in table 6, show that the resistance-lowering effect depends mainly on the total volume of the mixture of mucin or starch and pneumococci. It is clear that with increasing volumes of the infective mixture greater parts of the lungs are occluded and more lobes are involved in the inflammatory process. Such experimental infections have been produced by many authors in order to study the therapeutic effect of serum and chemotherapeutic agents on the course of experimental pneumonia.

Nungester and Kempf (60) observed that immune serum decreases the mortality rate and the incidence of pleurisy in rat lobar pneumonia produced by the mucin technic, but does not, by any means, suppress the latter. Continuing these experiments, Kempf and Nungester (36) showed that serum reduces the number of pneumococci in the blood but not the number in the lungs. When the administration of serum was delayed, invasion of the blood stream by cocci emanating from the focus of the infection occurred. Experiments on rabbit hemolytic serum showed that antiserum does not penetrate into alveoli to any demonstrable extent. Much more antiserum was required to neutralize the specific polysaccharides in the alveoli than the amount which had actually penetrated to that site.

Comparative studies on the effects of serum treatment and chemotherapy (15, 29, 30, 37, 92-94) with the aid of the mucin technic showed that in type I pneumonia immune serum is at least as effective as sulfapyridine, and a combination of serum treatment and chemotherapy (provided that combined therapy is started immediately after the infection) is more effective than either treatment alone. The enhanced effect is produced by a combination of the immediate immobilization of the cocci by sulfapyridine (92, 93) and of the slow bacteriostatic action of immune serum. In type III infections, sulfapyridine is more effective than immune serum, and the combination of serum and sulfapyridine

fails to reduce mortality below the rate observed when sulfapyridine alone is given.

In addition to its use in pneumococcal infections, mucin has also been employed in the establishment of virus infections of the lungs. Wheeler and Nungester (91) reported that with the aid of mucin they succeeded in producing

TABLE 6

*Effect of mucin and starch on experimental respiratory infections by Diplococcus pneumoniae*

TYPE	ANIMAL	PATHOGENIZING SUBSTANCE EMPLOYED	QUANTITY OF CULTURE INOCULATED	MORTALITY	REFERENCE
I	dog	Starch mixture (1 ml)	ml 0.01-0.03	5/60*	77
			0.04-0.1	16/38	
			0.25-0.6	15/25	
			1.0	3/6	
	dog	Starch mixture (6 ml)	0.001	1/6	77
			0.01-0.02	28/43	
			0.05-0.1	6/8	
			0.2-0.3	8/11	
	dog	Mucin (2 ml)	0.6	12/15	77
			0.25-0.5	6/8	
			1.0	24/29	
			3.0	10/10	
I	rat	Mucin	5.0	10/10	25
			0.02	16/32	
			0.1 × 10 <sup>-3</sup>	13/14	
			0.1 × 10 <sup>-4</sup>	24/24	
II	rat	Mucin	0.1 × 10 <sup>-5</sup>	11/11	15
			0.2 × 10 <sup>-3</sup>	15/15	
III	rat	Mucin	0.07 × 10 <sup>-3</sup>	12/14	14
			0.1 × 10 <sup>-3</sup>	14/14	
	rat	Mucin	0.1 × 10 <sup>-6</sup>	22/23	38
			0.5 × 10 <sup>-6</sup>	43/49	

\* The numerator shows the number of dead animals; the denominator shows the number of animals used in the experiment.

influenza pneumonia in hamsters. If the virus, alone, suspended in saline, was inoculated intratracheally only 9 per cent of the hamsters showed gross lesions of the lungs, whereas if the virus was suspended in mucin 71 per cent developed such lesions. However, mucin itself was not innocuous, since 25 per cent of the animals receiving mucin alone also developed gross lesions of the lungs.

## SUMMARY

Most of the observations made with mucin in intraperitoneal infections can be ascribed to a basic phenomenon: the coating effect of mucin on bacteria (64, 89). This effect of mucin provides an adequate explanation for further phenomena such as the inhibition of phagocytosis (89), the inhibition of intraphagocytic digestion (47, 59), the inhibition of antibody uptake (35, 69) and bactericidal action (24, 35, 47, 62, 66, 80). The other observations made on mucin are random and do not reveal the essential mechanism of their action. Thus, lethal effects were seldom observed (68), anticomplementary action is exerted mainly by granular substances (63, 64), inhibition of the appearance of leucocytes in the abdominal cavity (19, 62, 66, 67, 80, 82) is limited to the first hour after the injection of mucin and cannot be of overwhelming importance in infections by microorganisms which are eliminated mainly as a result of the extracellular bactericidal action of the peritoneal fluid (24, 62, 66, 80). The observations which led to the conclusion that the administration of mucin leads to an increased permeability of the barrier between the abdominal cavity and the blood stream (2, 52) can easily be explained by the coating effect of mucin on bacteria which, when injected into the blood stream, reached the abdominal cavity. Antigenic properties of mucin which enable it to act as a hapten and to neutralize the antibacterial antibodies were not revealed (52); and it has been shown that the degree of serological relationship of the mucin or of other carbohydrates to the infecting organism does not determine the degree of their activity (64). Without any doubt the coating action of mucin is also effective in infections of the respiratory tract, but in addition to its coating activity, it also occludes the smaller air passages and thus interferes mechanically with the normal eliminatory mechanism (31, 46, 77).

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## CELLULAR STRUCTURES AND FUNCTIONS CONCERNED IN PARASITISM<sup>1</sup>

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The investigations carried out by Dr. Avery and his school between 1913 and 1940 have provided the pattern, the master plan, used by our generation for the immunochemical study of infectious processes. His more recent publications, on the other hand, have dealt with problems which bid fair to constitute some of the dominant preoccupations of the workers of tomorrow: namely, the nature of those modifications of host chemistry which result from infection, and the mechanism by which hereditary characters are transmitted in microorganisms.

The mere recital of his many distinguished and varied contributions would be sufficient to honor him. It would, moreover, give us the vicarious enjoyment of many historical discoveries: the correlation of the virulence of pneumococci with the possession of a capsule; the recognition that not only virulence, but also the immunological specificity of the different pneumococcus types depends upon the chemical characteristics of the capsular polysaccharides; the development of rational and precise concepts and techniques for the production of protective antibodies and enzymes selectively directed against the capsular material; the demonstration that many carbohydrates other than those of pneumococci can determine immunological specificity—for example those constituting the capsules of *Klebsiella pneumoniae* or, even more interestingly, simple sugars like glucose and galactose when incorporated into synthetic antigens. Careful study of his writings would reveal a subtle awareness of the complexity of the infectious process. The participation of the different components of the bacterial cell in virulence, immunity, and allergy—the multiple response of the host expressed not only by the classical immune and allergic manifestations, but also by the production of abnormal proteins of significance as yet unknown—all found their place in his analysis of the course of experimental and natural infections. Throughout his career we could enjoy with him many excursions into problems of bacterial physiology: the recognition of the exacting nutritional requirements of *Hemophilus influenzae* for the X (heme) and V (cozymase) growth factors; the striking oxidation processes and autolytic mechanisms of pneumococci; and last, but not least, the spectacular demonstration that a desoxyribonucleic acid fraction extracted from a culture of capsulated pneumococci can transfer to non-capsulated variants and their progeny the hereditary property to produce the capsular polysaccharide of the former culture, thus causing a directed hereditary alteration of the cell by means of a soluble, purified cellular component.

<sup>1</sup> Based on the Oswald T. Avery Lecture delivered before the Society of American Bacteriologists. Minneapolis, Minnesota, May 13, 1948.



All these achievements and their extension by other workers to many groups of microbial species constitute in a surprisingly large degree the subject matter and the doctrine of modern medical bacteriology. It is of interest, therefore, to strive for a better understanding of the manner in which Dr. Avery's work has altered the course of our science in the past and is now affecting many of our efforts and viewpoints.

*Evolution of the concept of microbial parasitism.* Although the concept of parasitism appears obvious in general biology, its meaning in relation to microbial infections remains very confused even today. This confusion results in part from the historical fact that our knowledge of microbial parasitism has evolved from two unrelated sets of phenomena, caused by entirely different mechanisms: on the one hand, the attack on plants and animals by visible predatory parasites; on the other hand, the processes of fermentation and putrefaction in their various forms. Although the relation of contagion to miasms, fomites and specific particles had been postulated many times before the bacteriological era, it is doubtful that any of the early students of disease had really reached the concept that microscopic living agents could attack man or animal. It is worth recalling in this respect that, even after he had become convinced that the pébrine corpuscles bore a causal relationship to the silkworm disease, Pasteur entertained for two years the view that the corpuscles were not truly independent living agents but were produced by the silkworm itself (34). It is almost certain that the discovery that certain infections, favus or scabies for example, were caused by fungi or by insects—parasites which could be detected with simple optical means—helped to breach the gap between the abstract concept of contagion and the concrete fact of parasitism. But whereas one could see or imagine how the arthropod attacked the host, it remained out of the range of experience to visualize how a bacterium or virus could possibly do it.

The relation of fermentation and putrefaction to disease processes had also been postulated for many centuries. Suffice it to recall here the oft quoted statement by Robert Boyle that "he, that thoroughly understands the nature of ferments and fermentations, shall probably be much better able than he, that ignores them, to give a fair account of divers phaenomena of several diseases, as well fevers as others, which will, perhaps, be never thoroughly understood, without an insight into the doctrine of fermentation".

The view that disease might be analogous to some derangement of orderly fermentation became more plausible when Pasteur traced the "maladies" of wine, beer and vinegar to the invasion of fermenting fluids by foreign germs capable of displacing or interfering with the microbes engaged in the legitimate business of manufacturing alcohol or acetic acid.

The participation of a foreign microscopic agent in obvious cases of parasitism (as in scabies) and in the spoiling of fermentation, appears today as sufficient justification for grouping under a general etiological concept many otherwise unrelated phenomena and to speak of contagious diseases as caused by parasites. But one may imagine that the nature of the relation between the chemical alterations during fermentation, the damage caused by a parasitic insect, and

the various infectious processes, must have appeared very obscure to many biologists. Indeed some must have wondered whether the similarity implied in using the word parasitism to discuss infectious diseases, went much beyond formal analogy and had any operational usefulness.

The biologically minded student of infection probably found comfort in the discovery by Metchnikov of the phenomenon of phagocytosis. As stated by Duclaux, there was at first sight something incongruous in a conflict between an ox and a bacillus; the sizes are so different (34). Since, according to Metchnikov, the bacillus was not dealing with the animal as a whole, but with phagocytic cells of the same order of dimension as itself, the relationship appeared more plausible and sensible. It is now known, in fact, that intracellular parasitism occurs in a great number of infections caused by bacteria and protozoa and that it is the rule in infections caused by viruses and rickettsia. In consequence, many aspects of microbial parasitism can be studied at the cellular level.

Unfortunately, transfer of the phenomena of parasitism from the level of the whole animal to that of the individual tissue cells did not make easier the analysis of the problem in mechanistic terms; the nature of the weapons used by the microbe to attack its host remained as obscure. Interestingly enough, progress in this direction has not come from classical descriptive biology, but rather from the observation of pathological processes and from attempts to unravel their chemical determinism. Recognition of the activities and nature of bacterial toxins was the first step in the analysis of the mechanistic aspects of parasitism. The evolution of our knowledge of these toxins began with the demonstration of the gross pathological effects of culture filtrates capable of reproducing many of the manifestations of the natural diseases; it was followed by the isolation in more or less pure form of some of the chemical substances responsible for these effects; it has now entered the phase of analysis of biochemical mechanism of toxin action, ushered in by the identification of the toxin of gas gangrene with the enzyme lecithinase, and by the brilliant hypothesis formulated by Dr. A. M. Pappenheimer, Jr. to correlate the action of diphtheria toxin with the inhibition of the cytochrome-b system. Other aspects of the evolution of our knowledge of bacterial toxins have just been reviewed by Dr. A. Bernheimer (14). These recent advances warrant the hope that it will soon be possible to account in biochemical terms for the toxemia which accompanies many bacterial infections, and for the other physiological and metabolic disturbances which constitute disease.

Granted the importance of these phenomena for the understanding of the clinical and pathological aspects of infection, they fail to reveal the primary cause of parasitism. Among microorganisms, very few can cause infection, and the problem of how the pathogens differ from their non-infectious relatives has perplexed bacteriologists since the beginning of our science. During the late 19th and early 20th centuries, countless studies were carried out in the hope of detecting metabolic or other biochemical differences correlated with infectiveness, but all in vain. Thus, it was discouraging to find that the ability of microorganisms to multiply *in vivo* is not correlated with any known nutritional and

other environmental requirements. In this respect, pathogenic bacteria apparently vary in a haphazard manner. Among acid-fast pathogens, for example, the leprosy bacillus has not yet been grown *in vitro*, the Johne's bacillus requires growth factors of unknown chemical nature, whereas the most virulent tubercle bacilli grow readily and abundantly in simple synthetic media. From another point of view, no valid evidence has yet been obtained that resistance to the bacteriostatic and bactericidal agents, or to the enzymes, normally present in tissue fluids or cells, determines the ability of a bacterium to survive and multiply in the body of a given animal. Neither is the ability to produce toxins a differentiating character. Typhoid or dysentery bacilli, for example, are not known to differ in this respect from many other gram negative bacilli which are only saprophytic; among group A streptococci, none is known to produce a more powerful erythrogenic toxin than the N. Y. 5, Type 12 strain which is essentially avirulent for all known experimental animals; or again, virulent and avirulent variants of tubercle bacilli are equally able to cause the toxic manifestations of tuberculin allergy.

Failure to explain infectiousness in terms of available biochemical knowledge led several workers during the first two decades of the present century to return to a more biological statement of the problems of infection. This attitude is well expressed in Theobald Smith's classical essay "Parasitism and Disease" (1934) in which infection is treated from the point of view of the ecologist.<sup>2</sup> In broad biological terms, the ideal parasitism was there conceived as a state of equilibrium between the infectious agent and the infected species permitting survival of both.

"Parasitism is in a sense a compromise or truce between two living things, accompanied by predatory processes whenever opportunity is offered one or the other party. The universality of parasitism as an offshoot of the predatory habit negatives the position taken by man that it is a pathological phenomenon or a deviation from the normal processes of nature. The pathological manifestations are only incidents in a developing parasitism. As human beings intent on maintaining man's domination over nature we may regard parasitism as pathological insofar as it becomes a drain upon human resources. . . ."

Disease could then be considered as the multiple manifestations, conditioned by genetic and environmental factors, of this delicate equilibrium between invader and invaded host (76, 80).

This broad biological and ecological point of view has been extremely useful in the analysis of epidemiological problems, but it has contributed little to the understanding of the mechanistic aspects of parasitism. Nevertheless, some progress in this direction was made by analyzing host-parasite relationships in the general terms of the immunological reactions which are directed by the in-

<sup>2</sup> In the only long conversation which it was my privilege to have with Dr. Theobald Smith, he confided to me that his early studies on fermentation and other metabolic reactions had been motivated more by the hope of elucidating the nature of virulence, than by the necessity of working out diagnostic biochemical tests. It was after he had lost hope of reaching an understanding of virulence on the basis of existent chemical knowledge that he turned his attention to the broad biological laws of parasitism.

vaded host against the parasite as a whole. For example, one spoke of anti-typhoid, antiplague or anticholera antibodies to account for whatever resistance could be established against these infections. Furthermore, immunologists soon established that cultures of one given microorganism could elicit the production of several different antibodies; thus, a heat labile (H) and heat stable (O) antigen were recognized in several bacteria; antitoxic sera were differentiated from antibacterial sera; and the agglutinin absorption technique revealed that each bacterial cell constitutes an antigenic mosaic made up of several immunologically independent components. It was also suspected that certain bacterial constituents or products, loosely called aggressins, played a particularly important part in the phenomena of virulence. But despite this general awareness of the complexity of bacteria, the relation of the structure of the microbial agent to its ability to behave as a parasite had never been defined in precise terms. It is, I believe, the analysis of pneumococcus infections, carried out in Dr. Avery's laboratory, which provided the method by which antigenic structure, virulence, immunity and the other aspects of parasitism have been shown to be the expression of certain morphological and chemical characteristics of the microbial cells.

*The mechanism of parasitism in pneumococcus infections.* We shall not review the pioneer work of Neufeld and his school on specific pneumococcus immunity, of Toennissen on the carbohydrate nature of the capsule of Friedländer bacilli, and several other related studies, which constituted some of the material out of which Dr. Avery built the immunochemical laws which we are to consider now.<sup>3</sup> It is the bearing of these laws on the mechanism of microbial parasitism, and not the history of pneumococcus immunology, which is the object of our present discussion. The facts, and their relationships, can be outlined as follows.

a. There is a definite correlation between virulence of pneumococci and their possession of a capsule detectable by microscopic and immunochemical techniques. (4, 5).

b. The non-capsulated variants are more rapidly killed than the capsulated forms, both *in vivo* in the normal animal, and *in vitro* in mixtures of normal serum and leucocytes. The capsule participates in virulence by increasing the resistance of bacteria to phagocytosis. (4, 5).

c. The capsular substance is a polysaccharide. It varies in chemical structure from one pneumococcus type to another and conditions at the same time type specificity and virulence (4, 5, 46).

d. Specific antibodies directed against the capsular polysaccharides protect against infection by neutralizing the ability of the capsules to interfere with phagocytosis (4, 5); a similar result can be achieved with enzymes capable of hydrolyzing the capsular polysaccharides (6).

e. Although the reactive groupings of the capsular polysaccharides responsible for immunological specificity can survive many types of chemical and enzymatic treatment, the antigenic effectiveness of the complex polysaccharide antigen is

<sup>3</sup> A comprehensive survey of the historical aspects of this problem is given by B. White (86).

far less stable. In other words, the ability of capsulated pneumococci to elicit the production of antibodies protective against infection is optimum only when the bacteria used for immunization are prepared by techniques which maintain the antigenic integrity of the capsular structure (8, 11, 26, 27).

f. Immunization with pneumococci elicits the production of many antibodies directed against components of the bacterial cell other than the capsular substance (other polysaccharides, proteins, etc.) These other antibodies, however, have little if any ability to protect against infection although they may play a part in certain pathological processes, for example in those caused by allergic reactions to bacterial products (9, 47, 83, 84).

The significance of these facts can be emphasized from several different points of view. For many workers, the most important contribution made by Dr. Avery was the demonstration that carbohydrates play a part in virulence, antigenicity, immunity, and allergy equal to, if not greater than, the rôle classically attributed to proteins. Even more important, it seems to me, was the recognition that virulence and immunity can be analyzed apart from the parasitic cell as a whole, in terms of a few highly specialized components of it. In the pneumococci, these components were found to be the visible capsules, made up of polysaccharides; in other microbial species they might be other cellular structures, of another chemical nature. The important step, according to the view taken in the present discussion, was to have recognized the necessity, and the possibility, of analyzing infection not only in terms of general ecological host-parasite relationships, but in terms of identified cellular components of the parasite—its offensive and defensive weapons which effect the host and against which the host reacts.

This concept has now reigned over the study of infectious disease for a quarter of a century, and to enumerate its successes would be to recount the history of medical bacteriology during that period, an obviously impossible task. It is clear, however, that this approach does not constitute the only fruitful one to the study of infection; in fact, as was mentioned earlier, Dr. Avery himself has during the past ten years become vitally interested in other aspects of the problem of infectious disease, for example the altered chemistry and physiology of the infected host (1, 52), and the mechanisms of transmission of hereditary characters in microbial species (10, 57). Nevertheless, as the mechanisms which condition parasitism are the limited object of the present discussion, I shall attempt to discuss this problem further by applying the experience gained from the study of pneumococcus infections to the formulation of hypotheses concerning the pathogenesis of tuberculosis.

*Tuberculosis as an example of parasitism.* Tuberculosis illustrates well the problems of parasitism, because it presents a situation where the causative microorganism can live in apparent equilibrium with the infected host for prolonged periods of time, often causing only limited, or even no signs of clinical disease. The pathologist has described with extraordinary thoroughness the histological aspects of the response of the host to the parasite, from the initiation of the infection, to the establishment of the lesion, and its arrest or progression

to fatal outcome. The clinician has learned to recognize, predict and to some extent control, the manifestations of the disease. The epidemiologist has accumulated data describing the natural course of the disease in new and in immune populations, and the influence of environmental factors on morbidity and mortality. The bacteriologist has learned to grow the bacillus, and knows much of its peculiar chemistry. But despite this immense background of theoretical and practical knowledge of the disease and of the causative organism, little is known of the mechanisms by which tubercle bacilli become established in a new host, and cause disease, or of the processes used by the infected host to overcome the infection. In other words, we know much of the ecological aspects of host-parasite relationships in tuberculosis, hardly anything of the means used by the bacillus to behave as a parasite.

Of the many strains of acid fast bacilli which occur in nature, only very few can cause progressive disease in man or animals. The virulent forms exhibit a marked degree of specificity in their host range and have been classified on this basis into several pathogenic types: human, bovine, murine, avian, piscine. Nothing is known of the factors which determine specificity in host range.

Within any given pathogenic type, cultures of tubercle bacilli may vary greatly in virulence. This statement can be illustrated with a few specific examples of strains readily available to laboratory workers. The human strain H37 was isolated at the Trudeau sanatorium in 1905. In the course of time, two variants have been separated from it. One, H37Rv, is so virulent that a single or a very few cells can initiate progressive disease in guinea pigs (77) or susceptible mice (64); the other variant, H37Ra, is so devoid of virulence that even very large doses of it fail to cause progressive infection. Another strain, R<sub>1</sub>, was also isolated from a patient at the Trudeau sanatorium; this strain however, soon lost much of its virulence and became stabilized at a level such that it fails to produce progressive disease in normal guinea pigs although it can infect silicotic animals (78). From this somewhat attenuated strain, there has also been isolated a variant (R<sub>1</sub>Ra) which, like H37Ra, is so devoid of virulence that it cannot infect even silicotic animals. Other familiar examples of differences in virulence are the two bovine strains: Ravenel, which has remained highly virulent since it was first isolated some 50 years ago, and BCG which is sufficiently avirulent to permit its use as a living vaccine for immunization (19).

*The relation of virulent and avirulent tubercle bacilli to phagocytic cells.* Experimental pathologists have established many fundamental facts describing the comparative behavior of these different strains in resistant and susceptible animals. We shall consider for the present time only those facts which pertain to the behavior of the bacteria toward phagocytosis. Whereas, among pneumococci, the non capsulated organisms are much more readily phagocytized than the capsulated forms, no striking differences in rate of engulfment by phagocytes has as yet been described between virulent and avirulent variants of tubercle bacilli. Following their introduction into the animal (or in tissue cultures or other *in vitro* phagocytic systems) both the virulent and avirulent bacilli are rapidly taken up by polymorphonuclear leucocytes and find their way

into mononuclear cells a few hours later. It is the course of events subsequent to phagocytosis which differentiates the two forms of bacilli. The avirulent variants may survive intracellularly for prolonged periods of time, but fail to multiply to a significant degree. On the contrary, the virulent organisms rapidly increase in numbers in the susceptible animals and soon become detectable both within and outside the phagocytic cells. Whether bacterial multiplication occurs extracellularly, intracellularly, or both, is a question which has not been convincingly answered, and which cannot be discussed here.

During the past few months, my colleague, Dr. Hubert Bloch (17), has made a few observations which may contribute to the understanding of the relation of virulence to phagocytosis. By studying the exudate at intervals of time after injection of bacilli into the peritoneal cavity of mice, he could confirm that both avirulent and virulent forms were rapidly engulfed by phagocytic cells. He further gained evidence that engulfment of the virulent bacilli often resulted in the death and disruption of the phagocyte, a fact not observed with the non-virulent forms. These observations suggest the important conclusion that virulence is correlated with the ability of the bacillus to cause injury to the phagocytic cell. It seems legitimate to wonder whether this cytotoxic effect does not result in the liberation from the phagocytic cells of products that favor the further growth of the bacilli, a view for which suggestive evidence will be presented later.

*Morphological and chemical differences between virulent and avirulent tubercle bacilli.* Many efforts have been made to recognize between virulent and avirulent tubercle bacilli differences in their immunological or chemical characteristics, but with unconvincing results. On the other hand, bacteriologists have long been aware that certain morphological aspects of the bacterial growths appear to be correlated with virulence (18, 37a, 63). Without attempting to review earlier observations, I shall limit myself to a brief summary of the morphological characteristics recognized by Dr. Middlebrook in cultures of tubercle bacilli cultivated in the liquid and agar culture media developed in our laboratory (33, 59). In all virulent forms of human and bovine strains so far studied, the cells exhibit a marked tendency to adhere to one another in the direction of their long axis; this tendency results in the formation of strands of bacilli, which can be very long and at times extend over several microscopic fields, and which are several cells in thickness. In contrast to this serpentine pattern of growth, the avirulent variants exhibit either random growth or perhaps a rosette arrangement of the cells. These microscopic morphological differences are reflected in the macroscopic appearance of the cultures in liquid and on solid media, the virulent forms tending at first to give rise to thin films of growth spreading by the outward progression of the long strands of cells, whereas the non-virulent variants tend to multiply in the form of isolated islands consisting of heaped masses of bacilli (Plate I and Plate II). It is of interest that these fundamental morphological characteristics can be observed in animal tissues and therefore are not artifacts due to cultivation in unorthodox media (55). On the basis of present information, therefore, it appears worth considering as a working hy-

pothesis that the substance which tends to make the virulent bacilli adhere to each other and which causes their spreading mode of growth is in some obscure way correlated with virulence, perhaps by virtue of its ability to exert a toxic cytolytic effect on phagocytic cells.

Unfortunately, there is as yet no convincing information concerning the chemical nature of this substance and it can only be stated that the tendency of the virulent bacilli to grow in the form of "cords" is overcome by the addition to the culture medium of certain types of non-ionic wetting agents (33, 59). It is possible that the morphological differences between virulent and avirulent bacilli are not of a qualitative but only of a quantitative nature, and may be due to the production by the virulent forms of larger amounts of a certain hydrophobic substance, much less abundant in the avirulent form, which is wetted by the non-ionic surface active agents.

*Toxic and allergic reactions in tuberculosis.* Granted the ability of virulent tubercle bacilli to become established in susceptible hosts, knowledge of the nature of the disturbances by which they cause disease is far from clear and complete. The existence in tubercle bacilli of components capable of exerting a direct toxic effect has often been surmised, but has not been convincingly demonstrated (65, 68). The facts that undiluted tuberculin is somewhat toxic to cultures of normal tissues (2, 60, 66, 67, 69) and that killed bacilli, or extracts of them, can provoke the histological changes of tuberculosis (20, 71) are only questionable evidence of the existence of a toxin. There is no doubt on the other hand that tuberculous infections bring about a number of allergic reactions which are certainly of importance in the symptomatology and pathology of the disease. As is well known, the best studied among these is the allergy to bacillary protein which has been so extensively described under the name of delayed or tuberculin type of reaction. We need not review here the classical studies concerning the purified protein fractions which have been shown to possess a high degree of tuberculin activity (74). In addition to their great theoretical and practical interest, these purified proteins provide examples of convincing correlation between chemical nature and biological activity. It must be emphasized, however, that allergy in tuberculosis is not limited to the reactions to the purified proteins. Not only have other protein fractions been shown to exhibit tuberculin activity (56) but it is also certain that still other components of the bacterial cell (carbohydrates) can elicit allergic reactions of the immediate (anaphylactic) type (21, 56a). As far as is known, these reactions can be induced by virulent and avirulent bacilli alike. The capacity to induce the allergic state, therefore, cannot be synonymous with virulence, although it certainly plays an important part in pathogenicity.

It is one of the remarkable properties of tubercle bacilli that their mere presence greatly enhances the antigenicity of many unrelated substances (36). This is not a unique property of mycobacteria since, for example, antibodies against agar are often produced when other bacteria grown on agar gels are used for immunization. Nevertheless, the ability to enhance the antigenicity of diverse and unrelated substances is so intense in the case of tubercle bacilli that it must



have a bearing on the high level of allergy to their cellular constituents and products which accompanies tuberculosis; furthermore this property raises the possibility that immune reactions to some of the tissue constituents of the infected host may also take place during tuberculous infection. It is known that injection into rabbits, guinea pigs, and monkeys, of brain tissue mixed with heat-killed tubercle bacilli resuspended in paraffin oil brings about the production of antibrain antibodies and the development of allergic encephalitis (48, 61, 62). On the other hand, injection into rabbits of tubercle bacilli which have adsorbed certain synthetic surface active esters of oleic acid elicits the production of antibodies directed against these esters (58). As many types of surface active substances (phospholipids, cerebroside, etc.) are widely distributed in tissues, and can become adsorbed on tubercle bacilli, one may wonder whether allergy to tissue constituents altered by the infectious process does not play a rôle in the pathology of tuberculosis. Whether these speculations are justified or not, it remains a fact that certain peculiarities in the cellular structure of tubercle bacilli render them specially effective in enhancing the antigenicity of many types of natural and synthetic materials, and it is very gratifying that progress is being made towards establishing the nature of the bacterial components responsible for this interesting property (20, 67).

*Immunity to tuberculosis.* That a limited, but unquestionable level of immunity to tuberculosis results from prior exposure to tubercle bacilli, is shown by epidemiological and clinical observations in man, and can be demonstrated in experimental animals. But no convincing theory has been offered to account for the mechanism of this acquired immunity.

As tuberculous infection brings about the state of tuberculin allergy, one of

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#### PLATE I

1a. H37Ra. Ziehl-Neelsen stained smear of a 7-day-old culture in liquid medium containing 0.02 per cent 'tween 80' and 0.5 per cent serum albumin. Note the lack of orientation in the arrangement of the cells of this avirulent strain.  $\times 1000$ .

1b. H37Rv. Ziehl-Neelsen stained smear of a 7-day-old culture in liquid medium containing 0.02 per cent tween 80 and 0.5 per cent serum albumin. This culture was recently isolated from an experimentally infected mouse. Note the tendency to the formation of cords.  $\times 1000$ .

2a. H37Ra. 12-day-old culture on the surface of the agar medium containing 0.01 per cent tween 80 and 0.5 per cent serum albumin. The colonies are smooth surfaced, raised, and opaque.  $\times 90$ .

2b. H37Rv. 12-day-old culture on the surface of the agar medium containing 0.01 per cent tween 80 and 0.5 per cent serum albumin. The colonies are flat and translucent, and have serpentine markings.  $\times 90$ .

3a. H37Ra. 12-day-old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no tween. Note the non-oriented structure of the colonies: the colonies are heaped-up and have little tendency to spread out over the surface of the medium.  $\times 90$ .

3b. H37Rv. 12-day-old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no tween. The colonies have a serpentine structure; cords are visible in the form of loops at the thin undulate margins; and they are flat because of their tendency to spread out over the surface of the medium.  $\times 90$ .

The photographs were made by Mr. Joseph B. Haulenbeck.

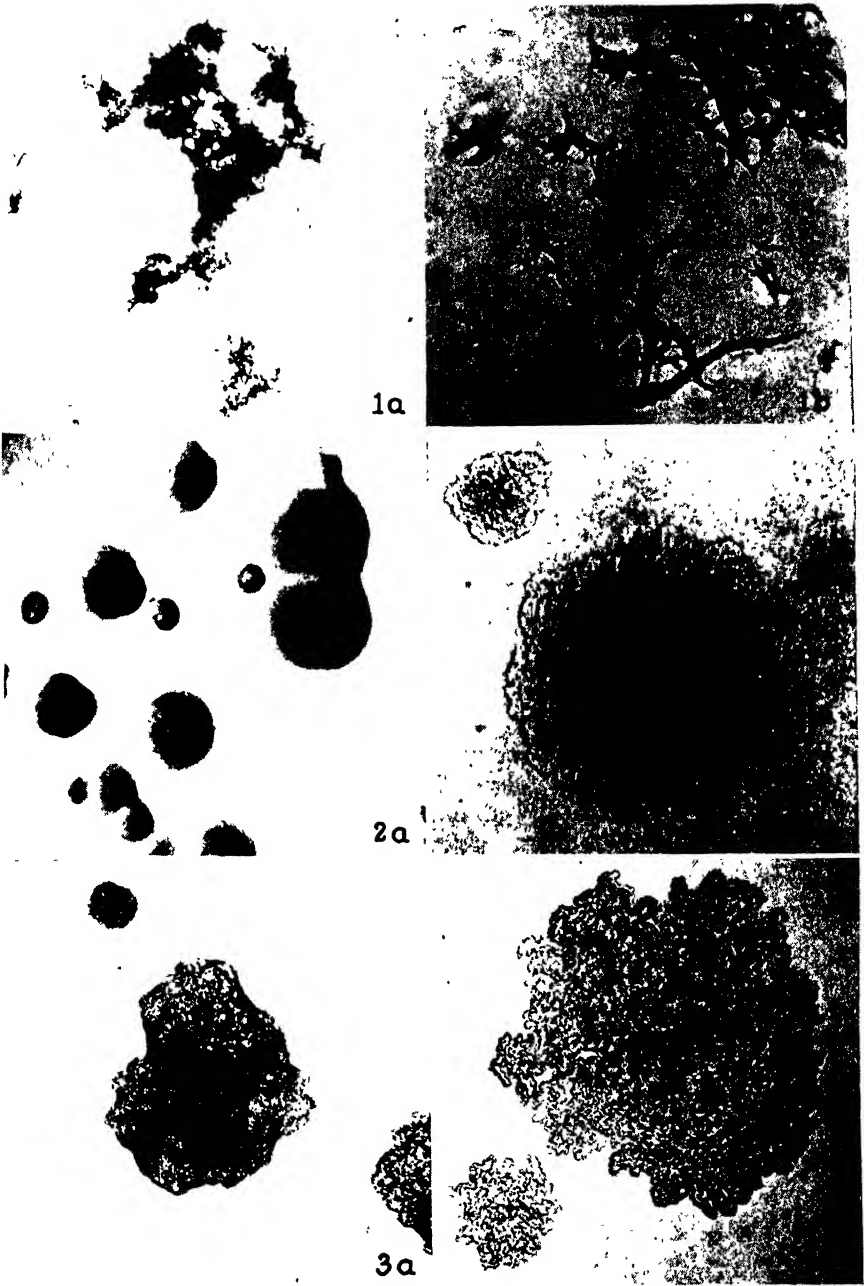


PLATE I

the possibilities which has been most widely discussed is that acquired immunity is a consequence, manifestation, of allergy. A brief statement of the known facts of allergy in pneumococcus infections may facilitate a more precise definition of this problem. As already mentioned, infection with, or injection of, pneumococci elicits multiple immunological reactions directed against the different antigenic constituents of the bacteria; several of these reactions have been recognized in terms of allergic manifestations. Thus, intradermal injection of the specific capsular polysaccharide into the infected or immunized animal calls forth an immediate, anaphylactic type of reaction, whereas injection of the bacterial nucleoprotein fraction or of the non-specific somatic polysaccharide (C) results in the delayed tuberculin type of reaction (12, 35, 47, 83). It is only in the case of the first of these three antigens (the capsular polysaccharide) that the allergic reaction depends on the presence in the serum and tissues of an antibody which is capable of protecting against infection; in other words, allergy to the capsular polysaccharide is correlated with a state of immunity. Whether in the case of pneumococcus, allergy means immunity, is therefore a question that can be answered only if one qualifies the nature of the bacterial antigen which is used to measure the allergic state. A similar situation may obtain in the case of tuberculosis. Although it has been shown that skin allergy (delayed type) to the tuberculoprotein does not necessarily reflect a state of immunity (65, 67, 68), it remains possible that there exist constituents of the tubercle bacilli against which is directed an immune reaction which can be recognized either by an allergy test or by immune resistance to infection. But, unfortunately, these bacterial constituents, and their corresponding immunological reactions, have not yet been identified.<sup>4</sup>

As the ability of the bacilli to exhibit the serpentine growth pattern seems to be correlated with virulence, it is enticing to believe that an antibody directed against the substance responsible for this mode of growth would have protective value. There is as yet no evidence in favor of this view; and in fact, the knowledge that immunity can be established by injection of the attenuated strain

<sup>4</sup> It should be pointed out at this time that the accumulation of inflammatory cells evoked at the site of the allergic reaction probably plays a rôle in modifying resistance, a fact which complicates the analysis of the relation of allergy to immunity.

## PLATE II

1. Ziehl-Neelsen stained preparation of an 8-day-old culture of avirulent tubercle bacilli (H37Ra), grown in oleic acid albumin medium. The bacilli are not oriented and form clumps.  $\times 1520$ .

2. Ziehl-Neelsen stained preparation of avirulent tubercle bacilli (H37Ra), grown in oleic acid albumin medium containing 0.5 per cent chick embryo extract. The bacilli are arranged in parallel and form cords.  $\times 1520$ .

3. Ziehl-Neelsen stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid albumin medium. The bacilli form cords.  $\times 1520$ .

4. Ziehl-Neelsen stained preparation of virulent tubercle bacilli, (H37Rv) grown in oleic acid albumin medium containing 0.5 per cent chick embryo extract. The cords are tighter than in fig. 3; the parallel arrangement is more pronounced.  $\times 1520$ .

The photographs were made by Mr. Julian Carlyle.



PLATE II

BCG would seem to militate against it. During the past few months, however, this objection has become less valid as a result of discoveries in the fields of pneumococcus and streptococcus immunity. There have been isolated a number of variant strains of these cocci which produce amounts of capsular polysaccharides (in the case of the pneumococci), or of the M proteins (in the case of streptococci) too small to allow bacterial proliferation *in vivo* and therefore too small to endow the bacteria with virulence, but sufficient to elicit the production of the homologous specific protective antibodies; in other words, there are known several strains of pneumococci and streptococci which have become stabilized at levels of production of the "virulence" antigens not adequate to permit progressive infection, but capable of calling forth the specific immune reaction (54, 70, 82).

It would be enlightening to reexamine from this point of view the attenuated cultures of anthrax, fowl cholera, and swine erysipelas used by Pasteur in his celebrated experiments on immunization in order to determine whether they had retained some of their "virulence" antigens. For the immediate purpose of our discussion, in any case, it is worth considering the possibility that tubercle bacilli can also be stabilized at levels where they produce enough of the virulence antigen to immunize, but not enough to produce progressive disease. Of special interest in this respect is the fact that ability to exhibit the serpentine pattern of growth is not an all or none property in tubercle bacilli. On the contrary, strains differ quantitatively rather than qualitatively with reference to it, and, by adequate cultural techniques, it is possible to recognize that, in BCG cultures, the bacteria retain to some extent the ability to organize themselves according to this pattern, suggesting the existence in BCG of a small residuum of the virulence property (59).

*Effect of in vivo environment on the antigenic structure of bacteria.* The BCG vaccine consists of living organisms and its effectiveness as an immunizing agent depends upon its ability to achieve a limited degree of proliferation *in vivo*. This fact raises the possibility that the bacilli produce during growth in the animal tissues an antigen which is not produced *in vitro* in the ordinary culture media. There is a well established precedent for this hypothesis. In confirmation of Bail's early claims on the production of aggressins, it has been demonstrated during recent years that the edema fluid in animals infected with anthrax bacilli contains an antigen which is not produced by the bacteria in ordinary bacteriological media; this antigen is formed *in vitro* only under very special cultural conditions (39, 45, 85). There is as yet no evidence for the occurrence of a similar phenomenon in the case of tubercle bacilli, and the fact that it is possible to establish with vaccines made up of heat killed virulent bacilli a level of immunity equal to that induced by BCG suggests that the immunizing antigen is readily produced by the bacilli *in vitro*. Nevertheless, recent observations suggest that the morphology of attenuated tubercle bacilli, and perhaps therefore their antigenic constitution, can be modified if not qualitatively, at least quantitatively, by growth in the presence of certain components of animal tissues. Thus, Dr. H. Bloch has found that addition of aqueous extracts of chick embryo

to oleic acid-albumin medium causes non-virulent tubercle bacilli to exhibit the serpentine pattern of growth characteristic of the virulent forms, and that the same extracts also affect the virulent bacilli in the same direction, inciting them to grow in the form of bacillary cords much tighter than those obtained in ordinary media (Plate II). No technique is available to translate these findings into immunological terms. Nevertheless, preliminary observations suggest that the chick embryo extract has an enhancing effect on some aspect of virulence of the organisms growing in its presence; this effect however is entirely reversible, as the bacilli return to their original level of virulence as soon as transferred to media devoid of the extract (16).

In the past, the effect of the *in vivo* environment has been considered chiefly from the point of view of classical immunity reactions. It is important to remember, on the other hand, that many metabolic phenomena which must play a part in pathogenicity, are markedly altered by environmental changes. It is sufficient to recall in this respect that slight modifications in the composition of the medium, which have little if any effect on the rate of bacterial growth, increase or decrease out of all proportions the production of toxins by diphtheria bacilli, clostridia and other pathogenic agents (14). Similarly, the production of certain enzymes is an adaptive process, and depends upon the presence in the culture medium of the homologous substrate (28, 38). Although only two enzymes of interest in pathology (hyaluronidase and the lecithinase  $\alpha$  toxin of clostridia) have so far been shown to exhibit adaptive behavior, the phenomenon is certainly one of wide occurrence and cannot fail to be of significance in pathological processes. The effect of the *in vivo* environment on the cellular structure and metabolic equipment of bacteria, and therefore on their pathogenic behavior, is one of the virgin fields of medical bacteriology.

*Factors which affect the proliferation of tubercle bacilli in vivo.* In the immunized or naturally resistant host, virulent tubercle bacilli may survive for prolonged periods of time, although they fail to increase in numbers. It is certain that under body conditions the bacilli respire and metabolize, but the nature of the bacteriostatic and bactericidal influences which control their multiplication is entirely unknown. Analysis of this problem will require eventually some knowledge of the metabolic reactions used by the bacilli for multiplication *in vivo*. As is well known, virulent tubercle bacilli can grow *in vitro* in synthetic media of extremely simple composition, but this tells us little of the substrates which they *do* utilize in the animal tissues. The observations made in our laboratory have led us to emphasize the rôle of lipids in the nutrition of these bacteria (29, 32), but it has not yet been proven that this rôle is as important in the body as we find it interesting in our test tubes. Nevertheless, there is no question that lipids play a peculiar rôle in the metabolism of tubercle bacilli. Thus it is a remarkable fact that, under the proper conditions, certain long chain fatty acids and alcohols, and even hydrocarbons, are much more effective than glucose or glycerol in stimulating their respiration (44, 51, 72). Furthermore, the yields of avian and human mycobacteria grown in serum albumin media containing palmitic, stearic or oleic acids (either in the form of the sodium soaps or of the water

dispersible esters), linoleic, linolenic or arachidonic acids, lecithin, kephalin, sphingomyelin or lignoceric acid, increase in direct proportion with the concentration of the fatty acid in the medium (29, 32).

Evidence is now accumulating that certain tissue components are capable of exerting a stimulating effect on the growth of tubercle bacilli. For example, the phospholipid sphingomyelin permits the growth of small inocula even in the absence of serum albumin and at the same time increases markedly the yield of growth within a given period of incubation (32). This dual effect can be analyzed in terms of two independent properties. On the one hand, sphingomyelin, like albumin, can detoxify long chain fatty acids and thereby exerts a protective effect on the bacilli. On the other hand, it acts as a source of nutrient probably by virtue of the lignoceric acid which is present in amide form in its molecule. Other tissue factors present in aqueous extracts of chick embryo, and as yet unidentified, have also been found to enhance and modify the growth of tubercle bacilli *in vitro* (16, 37, 76a). It appears likely, therefore, that as more is learned of the requirements of these organisms, it will become possible to reduce considerably their generation time *in vitro*, a result which would be of theoretical and practical importance especially in view of the fact that the generation time of tubercle bacilli *in vivo* appears under many conditions to be much shorter than is usually assumed.

Although serum is a poor medium for the growth of tubercle bacilli, their growth is abundant when the cellular elements of the blood have been lysed (66a, 88). These facts suggest the possibility that the growth promoting effect of sphingomyelin and of substances similar to those present in embryo extract may have a bearing on the problem of bacterial proliferation *in vivo*, particularly in caseous material and in the necrotic tissue found in silicosis. The relation of the cytotoxic properties of virulent tubercle bacilli to the local release of cellular components available for the nutrition of the bacteria is also a subject worth meditation and experimentation.

The inhibition of growth by immune processes directed against the metabolic enzymes of bacteria has often been postulated. "Antiblastic immunity" was first mentioned in the case of anthrax by Ascoli (3) and later in the case of pneumococcus infection by Dochez and Avery, possibly the basis of mistaken interpretation of accurate experimental findings (15, 25). The concept of antiblastic immunity is compatible with the knowledge that antibodies can be produced against certain enzyme proteins and can inhibit their enzymatic activity (75). The recent finding that, in paramecia, specific antibody not only abolishes motility, but also brings about a hereditary alteration of the antigenic structure of the cilia, (76b), demonstrates that immune reactions can affect the course of the enzymatic reactions which determine the composition of microbial cells. At the present time, however, factual evidence that there exist immune humoral factors which can inhibit the proliferation of parasites has been obtained only in the case of infection by certain protozoa and helminths (81). Whether the "anablastin" demonstrated by Taliaferro and his colleagues is really an antibody and whether it has its counterpart in tuberculous disease are questions which cannot be answered at the present time.

Finally there remains to be mentioned the possible existence in tissues of substances other than immune bodies capable of exerting a bacteriostatic or bactericidal effect on growth. Thus, long chain fatty acids are extremely toxic for tubercle bacilli, and although their toxic effect is overcome under ordinary circumstances by serum albumin and sphingomyelin (13, 22, 23, 24) there may be circumstances, in autolyzing tissues for example, where their bactericidal effect can come into play. Sphingosine may also be toxic for tubercle bacilli (32). In fact, it is becoming apparent that the resistance of tubercle bacilli to antibacterial agents has been greatly exaggerated. Not only are mycobacteria normally susceptible to many more agents than was formerly suspected, but it is also possible to increase their susceptibility by slight changes in the environment. Thus, all surface active substances which facilitate dispersed growth of tubercle bacilli by wetting their hydrophobic surface increase the susceptibility of these organisms to a variety of antibacterial agents (triphenylmethane dyes, *p*-aminosalicylic acid, streptomycin, subtilin, penicillins) (30, 49, 50, 79). For example, strains of mycobacteria which can grow in oleic acid albumin medium (in which their surface is strongly hydrophobic) in the presence of 100 micrograms of penicillin or of subtilin per milliliter, are inhibited by 5 micrograms of these inhibitors in media containing the proper wetting agent. Whether this is due to the more dispersed state of the cultures in media containing the wetting agents or to the fact that the latter substances facilitate access of the inhibitor by modifying the bacterial surface is as yet unsettled. In any case, the important conclusion can be drawn from these facts that the resistance of tubercle bacilli to inhibitors which are effective against other microbial species need not be the consequence of peculiarities in the metabolic equipment of the former organisms. It may be due merely to the hydrophobic character of their surface which retards or prevents the contact between the inhibitor and the susceptible cellular substrate. Although these results were obtained in artificial media, and with synthetic wetting agents, it is worth keeping in mind that animal tissues also contain many types of surface active agents which, under certain circumstances, may alter the susceptibility of tubercle bacilli to various agents.

Whatever the real nature of the mechanisms which hold in check the increase in numbers of tubercle bacilli in the infected individual, they often result in a delicate equilibrium between parasite and infected host. But this equilibrium is extremely unstable, and it is well known that many changes in the host or his environment can bring about reactivation of a dormant tuberculous infection. To illustrate this statement, it is sufficient to recall how suddenly tuberculosis mortality rates increased in different parts of the world during the first and second World Wars. Within a few months, tuberculosis had taken the lives of many individuals who, prior to the upheavals associated with the war, were living in a state of unsteady equilibrium with their disease (1a, 87). These observations provide fertile hunting ground for the epidemiologist. From our point of view, they emphasize once more the necessity of identifying those structures and reactions at the level of which the animal and bacterial economy influence each other.

*Conclusion.* We have travelled a long way from the analysis of the rôle played by capsular polysaccharides in the pathogenesis of pneumococcus infections, but



a single thread has led us through our wanderings. We have attempted to analyze the ability of microbial parasites to invade and cause disease, and the immunological and pathological responses of the infected hosts, in terms of the rôles played by the different constituent parts of the parasitic cell.

The results obtained in the study of pneumococcus immunity remain as a symbol of perfection and as a goal in our efforts. Even today, after twenty years of experimentation in all fields of medical microbiology, no experiment compares in elegance and convincingness with the demonstration that one can bring about specific immunity against pneumococcus infection by injecting the purified capsular polysaccharides into experimental animals (41, 42, 43, 73) or man (35, 53). Indeed, it has been found possible to establish a certain level of immunity against virulent pneumococci by means of synthetic antigens which mimic the immunological specificity of the capsular polysaccharides, although they do not contain any part of them (40). The analogy with the evolution of our knowledge of hormones and vitamins is worth bringing out at this time. Following the recognition by physiological methods of hormonal influences and vitamin deficiencies, the chemist, guided by precise biological tests, identified and synthesized the active chemical agents. A similar process is slowly emerging in the study of infectious diseases, and Dr. Avery's work has historical significance because of his pioneer and unexcelled contributions in this field. It was he, who first established experimentally the importance of identifying the structures and functions of microbial cells which are of significance in the infectious process. With him, microbial parasitism evolved from an ecological concept into a body of facts and doctrines which define in physicochemical terms the mechanism of host parasite relationships.

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# PROPERTIES OF CERTAIN RAPIDLY ACTING BACTERIAL TOXINS AS ILLUSTRATED BY STREPTOLYSINS O AND S<sup>1, 2</sup>

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The nature of bacterial toxins, their mode of formation and action, and their rôle in the genesis of disease, are problems that have occupied investigators since the early days of medical microbiology. With the recognition that poisonous bacterial products could account for the major manifestations of each of a number of diseases, there developed an active and enduring interest in the qualities of the poisons themselves. In particular, the toxins of the diphtheria, tetanus, and botulinus bacilli have been most intensively investigated.

Apart from the classical exotoxins just mentioned, there exists a rather large group of toxic bacterial products which, on the whole, have been studied less thoroughly. Because of the diversity of their effects, it is difficult to define them, as a group, in a completely satisfactory manner. The toxic bacterial products to be discussed differ from the classical exotoxins in several ways, one of the most striking of which is that their visible effects are not preceded by a latent period of appreciable duration. They act swiftly, and might therefore be termed *rapidly acting* or *acutely acting* toxins. It is noteworthy also that most, if not all, of the rapidly acting toxins are hemolytic, a property which is not shared by diphtheria, botulinus, or tetanus toxin (tetanospasmin). Although none of the rapidly acting toxins has been isolated with certainty as a pure substance, it is clear that the lethal activity of the most potent preparations is of a much lower order than that of the classical exotoxins. Rapidly acting toxins are produced by some strains of staphylococci and streptococci, by pneumococci, by several of the clostridia, and undoubtedly by many other bacterial species.

Among the pathogenic bacteria, a single species may elaborate several distinct toxins of which one or more is of the rapidly acting type. This is true of many strains of *Streptococcus pyogenes*, a fact which has been clarified especially by Todd (23) who differentiated by immunological means, two hemolytic toxins which he designated streptolysin O and streptolysin S. These two substances are, in certain respects, representative of the class of rapidly acting toxins, and in the following discussion they will be considered in some detail. They differ from each other not only immunologically and in the manner in which they are affected by certain organic substances, but also in the conditions governing their formation.

Before discussing the factors involved in formation of streptolysins O and S, it is pertinent to mention some of the conditions necessary for optimal growth of

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the organism. Nutritionally, the pathogenic streptococci are among the most exacting of all bacteria. In addition to salts and an energy source such as glucose, they require an impressive assemblage of amino acids and vitamins (27, 19, 8, 7). However, even when all of the substances known to be required for multiplication are present, growth in terms of bacterial weight per unit volume of medium is likely to be slight compared to that which can, under suitable conditions, be obtained. By increasing the concentration of certain of the nutrients, some of which had been found to be limiting, by reducing the total salt concentration to a minimum, and by neutralizing with alkali the lactic acid formed from glucose, there was evolved a medium of essentially defined composition capable of supporting growth amounting to several grams of streptococci, dry weight, per liter (8, 7).

The mere fact that a toxigenic bacterial species can grow under a particular set of conditions gives no assurance that it will produce toxin under the same circumstances. In fact, there does not seem to be any way of predicting whether the formation of a particular toxin will occur automatically as a consequence of growth. Streptolysin O was found to be produced in the defined medium just as it is in broth, and it appears, therefore, that its formation does not require extra-nutritional factors. The situation in regard to streptolysin S, however, is quite different. Its formation is favored by the presence of serum, either in growing cultures (23) or in contact with resting cells (25, 26), and until recently it was believed that appreciable amounts of streptolysin S are not formed in the absence of serum.

There are certain original and important observations on the formation of streptolysin, made by Okamoto, Itô and their coworkers in Japan that are not well known in this country. In 1939, Okamoto (17) discovered that yeast nucleic acid causes the formation of a very potent hemolysin in cultures of *Streptococcus pyogenes*. The striking effect of yeast nucleic acid can be demonstrated in blood agar plates as well as in broth cultures. The beta hemolytic zones around colonies growing on the surface of blood agar containing sodium nucleate are relatively enormous compared to those of colonies growing on plain blood agar. Although high concentrations of yeast nucleic acid improve the growth of streptococci, it can be shown that the great increase in streptolysin formation seen in plates or in liquid media is not dependent upon increased growth. The yeast nucleic acid effect appears to be specific for beta-hemolytic streptococci, as indicated by the findings of Okamoto (17) who studied several other species of gram positive cocci, and by the results obtained in our laboratory in which a wide variety of gram positive and gram negative organisms were examined. With T. H. Horrigan and H. H. Balch, we have observed the nucleic acid effect not only in strains of Lancefield Group A but also in certain strains belonging to groups D, E, G, H, and L. These findings do not imply that the nucleic acid effect is shown by all strains of these groups nor that it may not be encountered in strains belonging to other Lancefield groups.

The failure of certain agents, such as atmospheric oxygen, sulfhydryl compounds, and cholesterol, to affect the activity of the nucleic acid hemolysin

shows that it cannot be streptolysin O. In all respects studied, however, the nucleic acid hemolysin is identical with streptolysin S. The almost unlimited capacity of bacteria to undergo variation provided a means for testing further the possible identity of the nucleic acid hemolysin with streptolysin S. From a culture of Group A streptococci, it was possible to isolate a mutant which, unlike the parent strain, failed to produce streptolysin S. In broth cultures as well as in plates, nucleic acid, although having its usual effect on the parent strain, failed to stimulate lysin production by the mutant. In addition, examination of a series of strains, some of which produce only streptolysin S, others only streptolysin O, and others both, showed that the nucleic acid effect is demonstrable only in strains having the potentiality for streptolysin S formation. From these results, and the ones already cited, it can be concluded that the nucleic acid hemolysin is streptolysin S (9).

The capacity of ribonucleic acid to induce the formation of streptolysin S is possessed not only by yeast nucleic acid but also by ribonucleic acid from certain other sources, such as mammalian liver, wheat and bacteria. A single preparation of ribonucleic acid from tobacco mosaic virus, tested under the same conditions, failed to cause streptolysin formation—a finding which suggests a difference in structure between the virus ribonucleic acid and that from other sources. Desoxyribonucleic acid, in contrast to ribonucleic acid, appears to be inactive. Negative results were likewise obtained upon testing the products of acid- and alkali-hydrolyzed ribonucleic acid as well as the constituent purine and pyrimidine nucleotides and some of their hydrolysis products.

The streptolysin-forming action of some preparations of ribonucleic acid is markedly and specifically increased by treatment with ribonuclease, the activation, in some instances, being as great as ten-fold. Although different preparations of ribonucleic acid may vary considerably in activity, after they are treated with crystalline ribonuclease they show approximately the same activity, indicating that they possess approximately equal potential capacity to induce streptolysin formation.

On fractionation of the digestion mixture following the action of ribonuclease upon yeast sodium nucleate, it is possible to isolate a polynucleotide which is approximately one hundred times as active as untreated yeast nucleic acid. The active polynucleotide contains a greater proportion of purine nucleotide than is present in the starting material, but its exact composition has not been determined.

The biological significance of desoxyribonucleic acids is clearly established by Avery, MacLeod, and McCarty's demonstration (1) of the ability of desoxyribonucleic acid to direct, in a specific and inheritable manner, the synthesis of pneumococcal polysaccharide. Although ribonucleic acid is commonly believed to perform essential functions in living systems, there are no instances in which its rôle has been clearly defined. Streptococci furnish, so far as we know, the only physiologically specific test for a ribonucleic acid. Further study of what may appear to be but a laboratory curiosity will, perhaps, provide an insight into the function of ribonucleic acid, its structure, or both.



In addition to ribonucleic acid, or a fraction thereof, at least one other factor is essential for the formation of appreciable amounts of streptolysin S in cultures (9). This factor is present both in peptone and meat infusion, but is absent from the defined medium supplemented with polynucleotide. Study of its properties has shown that it can be replaced by minute amounts of maltose or by somewhat larger amounts of glucosamine. With the exception of trehalose, which is about one-fifth as active as maltose, other disaccharides, including the beta-glucosidic isomer, cellobiose, possess less than 10 per cent of the streptolysin-forming activity of maltose. As little maltose as M/64,000 can cause significant streptolysin formation in the presence of about 180 times as many glucose molecules. Essentially nothing is known of the mechanism whereby such relatively minute amounts of maltose exert their effect. Although observations like this appear to take us rather far afield, they may eventually contribute to the solution of other problems such as the mechanisms of formation and action of the toxin.

The chemical nature of the streptolysins has been the subject of a number of investigations. Both products are very labile substances of high molecular weight. Highly purified preparations of streptolysin O, obtained by Smythe and Harris (21), Herbert and Todd (12), and ourselves (4), exhibit the properties and elementary composition of proteins. Streptolysin O contains a relatively large amount of sulfur which occurs either in disulfide or sulfhydryl form, and it is activated by substances which reduce disulfide links to sulfhydryl groups. It is destroyed by proteolytic enzymes, and there is little reason to doubt that streptolysin O is a protein. Evidence of close chemical and biological similarity between streptolysin O and pneumolysin is indicated by the work of various investigators (15, 16, 20, 11 *et al.*). That streptolysin O is immunologically related to pneumolysin, tetanolysin and *Clostridium welchii* theta toxin, has been shown by Todd (22, 24).

The chemical nature of streptolysin S is more obscure. Herbert and Todd (13) fractionated filtrates of serum-broth cultures, and obtained a product which suggested that streptolysin S is, or is associated with, a lipoprotein. Okamoto and co-workers (18) fractionated filtrates of nucleic acid broth cultures, and obtained preparations of great potency, of which the most active was largely or exclusively polynucleotide in nature, and said to be free from protein. Finally, we (4) have fractionated filtrates of defined medium cultures containing maltose and a fraction of yeast nucleic acid, and have obtained a very potent product whose properties suggest that streptolysin S may be a protein or a nucleoprotein. Further work is needed.

Having considered in some detail the nature of the streptolysins and the circumstances attending their formation we may now proceed to some of their biological properties of which by far the best known is the capacity to lyse blood cells *in vitro*.

Study of hemolysis caused by rapidly acting toxins, including streptolysins O and S as well as lytic agents from other sources, has revealed rather striking similarities and differences in the kinetics of the hemolytic reaction—findings which must in turn reflect similarities and differences in the mechanisms of cellular injury (3). For example, rate of hemolysis, under a particular set of experimental

conditions, is directly proportional to concentration of lytic agent when the latter is streptolysin S or any one of several other rapidly acting toxins—a result that would be expected if these substances function catalytically, that is, as enzymes. Results entirely different from these are seen when the lytic agent is saponin, taurocholate, or tyrocidine, and still other results when it is streptolysin O. Other studies concerned with the effect of temperature on the kinetics of hemolysis have provided an independent means of classifying rapidly acting toxins, and have shown in addition, that these substances possess activation energies most of which fall within the range of those of enzymes.

Although it is self-evident that the substances under discussion act upon erythrocytes in ways that lead to the liberation of the cell contents, the nature of the initial reaction and that of the sequence of events that follow are relatively obscure. A partial but important exception to this statement is found in the discovery of MacFarlane and Knight (14) that the alpha toxin of *Clostridium welchii* specifically catalyzes the hydrolysis of lecithin. This toxin is hemolytic and generally cytotoxic, presumably because it destroys the lecithin of cell membranes.

Analysis of the *Clostridium septicum* hemolytic system (2, 3) indicates that lysis is preceded by two phases, the first of which is an irreversible alteration of erythrocytes caused by the toxin. This phase is specifically inhibited by antibody. In the second phase, the altered cells undergo swelling until they burst. The second phase, which is inhibited by sucrose but not by antibody, can be explained as resulting from loss of selective permeability, which leads to a kind of osmotic hemolysis. The hemolytic action of streptolysin S, although not studied as intensively, shows many of the features of the *Clostridium septicum* system. That of streptolysin O, however, appears to be fundamentally different; the stage of swelling is either lacking or lasts for such a short time that it escapes observation.

It is evident that the *initial* actions of streptolysins O and S differ fundamentally from each other and from those of certain other lytic agents. The rapidly acting toxins can be visualized as destroying specifically one or another linkage of the lipids, proteins or complexes thereof, which comprise the surface of the erythrocyte. Knowledge of the chemical nature of the initial change, that is, of the particular linkages attacked, should help elucidate the molecular architecture of the red cell membrane.

Because of the ease with which their presence can be detected using erythrocytes, the rapidly acting toxins just discussed are commonly designated as hemolysins. This term, however, is not altogether desirable because it implies that the action of the substances under consideration is limited to blood cells. As will now be shown, this is not the case.

Injection of partially purified preparations of streptolysin O into mice causes a fatal toxemia. Since the injected mice do not die of intravascular hemolysis, but rather of cardiovascular and perhaps other injuries, it is clear that cells in addition to those of the blood are attacked. Another indication that the action of streptolysin O is not limited to erythrocytes is shown by unpublished work of A. Brittis in our laboratory, who has found that streptolysin O, and indeed also other rapidly acting toxins, cause *in vitro* the dissolution of lymphocytes prepared

from mesenteric lymph nodes. The findings suggest that many or most substances that are erythrocytolytic are also lymphocytolytic.

A different approach to the action of streptolysin O has been provided by studying its effects on the isolated heart of the frog, washed free of blood, and filled with Ringer's solution. A heart prepared in this way will continue to beat approximately 35 times each minute for a great many hours provided it is supplied with oxygen. It has been shown (5, 10) that a single administration of partially purified, and considerably diluted, streptolysin O has little or no apparent effect. When, however, the first dose of streptolysin O is removed, and the heart washed twice with Ringer's solution and then treated with a *second* dose of streptolysin O identical with the first, the heart quickly stops beating. Application in a single dose of the total amount of streptolysin O given in two doses does not cause cardiac standstill. Two doses must be used, the first dose *sensitizing* the heart to the second. Sensitization occurs because the first dose releases from the heart tissue (and thereby deprives it of) a substance which inhibits the toxic action of streptolysin O. The protective substance derived from the tissue is removed in the perfusate when the heart is washed with Ringer's solution. This substance inhibits not only the systolic contracture-producing action of streptolysin O but also the lethal effect for mice. The inhibitory effects of the protective substance appear to be specifically directed against streptolysin O and closely related toxins. It would be of great interest to know what this inhibitor is but study of its chemical nature has been seriously hampered by the very minute quantities that can be conveniently obtained.

Whether the mechanism observed to operate in the frog's heart functions also in the mammalian heart is not certain, but there are observations which suggest that it does (6). Injection into mice of a just sublethal dose of streptolysin O causes the mice to become refractory to the effect of a subsequently administered lethal dose of the same substance. The refractoriness, which is not due to antibody, develops within six hours and lasts less than forty; it may well depend upon the liberation into the bloodstream of a toxin inhibitor similar to that released by the isolated frog's heart. In any event, it is notable that there exists in the mouse, and presumably in other mammals, an immune mechanism which is distinct from that underlying classical antitoxic immunity.

In conclusion, we have attempted to review some of the distinguishing features of streptolysins O and S, bearing in mind that they represent a class of biologically active substances distinct from the better known exotoxins. While these bacterial products do not constitute a field of study that is new, it is nevertheless true that many of the most basic questions concerning them have been only partially answered. Many of the other members of this class have been very lightly explored, but enough is known to indicate that the investigator who studies them will not be disappointed in what he sees, for they are almost as varied in their effects as bacteria themselves.<sup>3</sup>

<sup>3</sup> Many persons have contributed to the work which has been discussed. The author is particularly indebted, for collaborative experimentation, to Dr. G. L. Cantoni, Long Island College of Medicine, and in less tangible ways to his associates at New York University College of Medicine.

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# CHARACTERISTICS OF PATHOGENIC SPIROCHETES AND SPIROCHETOSSES WITH SPECIAL REFERENCE TO THE MECHANISMS OF HOST RESISTANCE

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That the spirochetes are among the neglected and forgotten children of the family of pathogenic microorganisms is reflected in the meagre information available on the biology and chemistry of these organisms. It is not surprising, therefore, that the mechanisms of host resistance in spirochetal infections are poorly understood. This situation is attributable partly to the difficulty in cultivating pathogenic spirochetes, the chronicity of some of the spirochetoses, and the apparent rarity and consequent lack of interest in certain spirochetal diseases in some parts of the world. Recently the ability of physicians successfully to treat leptospirosis, relapsing fever, and syphilis with penicillin and other chemotherapeutic agents has, unfortunately, forced fundamental studies of these organisms and the mechanisms of resistance into the background. It is desirable to have a fundamental basis for the treatment or at least understanding of cases of these diseases refractory to chemotherapy. Also, a study of this group of relatively rare diseases and disease-producing agents may shed some light on the

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mechanisms of more common or related diseases and organisms. Henrici has wisely said (74): "It is a truism of science that a study of rare and curious events in nature often brings to light general phenomena or principles which may be exaggerated in the rare but overlooked in the commonplace."

A valuable aid for the further study of antispirochetal resistance is the analysis and synthesis of the available data on the subject. The author, aware of no critical, up-to-date survey of the material on spirochetal resistance, has attempted to prepare such a review. Rigorous selection of material was necessary, as there is a great deal of published information on the individual spirochetoses, much of it in monograph form (25, 34, 78, 101, 142, 143, 150, 151, 204, 219, 225). In general the data are confined to leptospirosis<sup>2</sup>, relapsing fevers, and syphilis as the major spirochetoses, but pertinent facts on the other spirochetal infections occasionally are introduced<sup>3</sup>. *Treponema pallidum* and related organisms causing chronic granulomatous diseases are considered together with the *Borrelia*s and *Leptospira*s which produce acute diseases. The inclusion of the syphilis spirochete is justified by its many similarities to the other groups of spirochetes, aside from morphology. These similarities are described in later sections.

Although the histological changes in the spirochetoses, particularly syphilis, may enable the pathologist to distinguish one disease from the other, description of the pathological picture in these diseases has been kept down to a minimum in the review. The author considers it dangerous to use this picture alone as evidence upon which to base a decision as to whether a life cycle of spirochetes exists or as to the mechanisms of infection or host resistance. In so far as pathological studies contribute to the elucidation of the fundamental mechanisms of pathogenesis and host resistance in the spirochetoses, an effort is made to incorporate them in this review.

It is hoped that this review will provide a stimulus to research in antispirochetal resistance by furnishing a survey of past research and discussion and by pointing out possible pathways of future investigation. An ulterior motive, perhaps, is to stir up interest in the spirochetes themselves by stressing their qualities apparently unique among microorganisms.

#### I. MICROBIC FACTORS THAT MAY INFLUENCE HOST RESISTANCE

It is very difficult to divorce host from microbic factors in resistance to disease. Some of the host reactions may eventually be shown to be reflections of certain chemical and physical characteristics of the organisms. This interdependence

<sup>2</sup> Unless otherwise indicated, data for leptospirosis or leptospirosis refer to the species *Leptospira icterohaemorrhagiae* and diseases of man and animals caused by this species.

<sup>3</sup> The reader unacquainted with spirochetes and spirochetoses is referred to any standard textbook of bacteriology for a brief description of these organisms and diseases. Following are the genera and major species of spirochetes and the diseases they cause: *Treponema pallidum*—syphilis; *Treponema cuniculi*—rabbit venereal spirochetosis; *Treponema pertenue*—yaws; *Leptospira icterohaemorrhagiae*—infectious jaundice of Weil's disease in man and animals; *Leptospira canicola*—leptospirosis in dogs and man; *Borrelia duttoni*, *novyi*, and *recurrentis*, African, American and European relapsing fevers, respectively, in man; *Borrelia anserinum*—avian spirochetosis.

has been well expressed by the eminent French immunologist, Bordet: "Immunity is the organism's virulence to the microbe, just as virulence is the microbe's immunity to the organism."

### A. Morphology and life cycle

The manner in which morphology of spirochetes may affect phagocytosis of the organisms is discussed in section II D.

Striking morphologic changes in spirochetes, presumably due to the action of agglutinating or lytic antibodies, have been observed in the course of natural and experimental infections or *in vitro* upon contact of organisms and specific antibodies (18, 62, 78, 85, 101, 106, 219). The organisms tend to become agglomerated, lose their motility, and appear granular. These alterations in morphology have been associated with a reduction or loss in virulence of the organisms (18). Agglutination and granulation of spirochetes may occur also in degenerating cultures in the absence of antibodies (185, 208, 219). The granules appearing spontaneously *in vitro* have sometimes been considered not to be the result of degeneration but homologous to bacterial spores, providing a means of maintaining life under adverse environmental conditions (78, 131, 208, 219). Recently, investigators using the electron microscope have observed spheroidal bodies at the sides of intact *T. pallidum* and have called them asexual reproductive bodies (146). Similar bodies have not been seen in electron microscope studies of *Leptospira* (141) or *Borrelia* (122). Other workers have described *in vivo* experiments which may be explained by the postulation of a granular or infra-visible stage in a spirochetal life cycle (78, 131, 219, 230). Still others believe that *Borrelias* may have a developmental life cycle in their vectors, lice and ticks (190), including an infra-visible granular form. However, despite the impressive array of scientists supporting a granular form as part of a life cycle (78, 219), the evidence for such a stage or life cycle is not altogether persuasive. This is regrettable as this interpretation, if correct, might aid in the understanding of enigmatic aspects of latency, periodicity, and antibody and drug-fastness in spirochetal infections.

### B. Electrokinetic potential<sup>4</sup>

Most bacteria possess a negative electrokinetic potential at or near pH 7 (1, 48). However, there is no general agreement on the sign of the surface potential of pathogenic spirochetes (56). Part of the confusion undoubtedly has arisen from the lack of standardization of technique, age and dissociative state of organisms, the growth and suspending mediums in determination of the potential. Kligler and Aschner (98) stated that as a rule spirochetes and protozoa have a positive charge at the pH of blood in contradistinction to bacteria which are generally negatively charged at this pH (1, 48). If substantiated, this is a very basic distinction, as it would imply a fundamental difference in the chemical composition of at least the surface of the spirochetal or protozoan cell as compared with that of the bacterial cell.

<sup>4</sup> Also termed zeta or surface potential, or, loosely, electric charge.



Kligler and Aschner (98) reported, on the basis of cataphoretic studies, that *L. icterohaemorrhagiae* possessed a positive charge at the pH of blood. Timmerman (206) found these organisms to be positively charged in respect to dilute rabbit serum in which they were grown. Other authors have claimed that *Leptospira* is negatively charged (56). Brown and Broom (14) studied the effect of negatively charged colloids on *Leptospira* in the presence of immune and normal serums. They observed that the negatively charged spirochetes adhered to bacteria when treated with specific immune serum containing complement. Adhesion did not occur in the presence of normal serum. Negatively charged colloids could replace the bacteria; and when the colloid was, for instance, a copper sol, the organisms were killed.

Similar divergent observations as regards surface potential are recorded for *Borrelia* and *Treponema* (56). Culwick and Fairbairn (42) claim that *Borrelia recurrentis* may have "electrical variants" so that some strains are positively, others negatively charged. However, their technique for determining the surface potential is open to criticism. They examined stained films of infected blood and recorded the percentages of spirochetes adhering to red blood cells and the percentage not sticking. Presumably the spirochetes which adhered to the negatively charged erythrocytes were positively charged; those which did not were negatively charged. However, in careful cataphoretic experiments with various strains of trypanosomes, Brown and Broom (15) found that some of them carried positive and others negative charges. With the same strain a change in charge was observed after relapse and after treatment with arsenicals (16).

In section II B the possible relationship of zeta potential to ability of organisms to pass through the walls of the cerebral blood vessels and the significance of this observation are discussed. The possible influence of electrokinetic potential on the phagocytability of spirochetes is considered in section II D.

### C. Motility and sedimentability

Motility and sedimentability of spirochetes may influence the process of phagocytosis of the organisms by host cells. The point is taken up in section II D.

### D. Cultivation and preservation

Of the three genera of pathogenic spirochetes, the *Leptospira* are the most readily cultivated *in vitro* (208). *Borrelia* is very difficult to cultivate in initial culture and cannot consistently be maintained in serial passage. Although there are many so-called culture strains of *T. pallidum*, it is doubtful whether virulent strains of the organism have ever been cultured successfully *in vitro* (63, 93). *L. icterohaemorrhagiae* (140) and *Borrelia* (152) have been grown without loss of virulence in the chick embryo. Wile and Snow (231) have reported experiments suggestive of proliferation of *T. pallidum* in the embryo, but their evidence is not conclusive. *T. pallidum* has not been grown in tissue culture (94), a technic often successful for the growth of organisms which are not cultivable on lifeless media.

All of the mediums which have proved satisfactory for the cultivation of patho-

genic spirochetes have contained native animal protein in the form of blood, serum or ascitic fluid (99, 101, 147, 150, 208, 219). Whether the protein acts chemically as a nutrient or physically as a protective colloid against noxious substances is unknown. It is known that soaps on glassware are rapidly lethal to *Leptospira* (194). The protein may protect the organisms against fatty acids as albumin protects tubercle bacilli and other mycobacteria from harmful fatty acids in culture (49). *Leptospira* and *Borrelia* are obligatory aerobes (150, 208).

Chang (30) has recently found that the virulence of *L. icterohaemorrhagiae* may be preserved *in vitro* if a small amount of emulsion of fresh guinea pig liver is added to the medium. The mechanism of this effect is unknown.

The pathogenicity and parasitic ability of spirochetes may be correlated partially with their cultivability and growth requirements *in vitro*. *T. pallidum* which multiplies with great difficulty or not at all *in vitro* or in the chick embryo, is a typical tissue parasite which produces chronic manifestations similar to those of tuberculosis. *Borrelia* is principally a blood parasite, whereas *Leptospira* invades both blood and the tissues. Recent studies indicate that the hemoglobin or hematin of red blood cells is necessary or helpful for the cultivation of *Leptospira* (31, 163, 177, 185) and certain species of *Borrelia* (99). This requirement might explain in part the affinity of these organisms for the blood stream of the host. The greater propensity for multiplication might enable the *Leptospira* and *Borrelia* to proliferate more rapidly *in vivo* and to produce a more acute infection. Such an infection might evoke a more acute host reaction and hence a more prompt termination, fatal or otherwise. The more fastidious growth requirements of *T. pallidum* probably restrict its growth *in vivo*, resulting in a more lesisurely development of the infection and consequently of host reaction to it.

Levaditi and Vaisman (116) found that intratesticular injection into rabbits of *T. pallidum* suspended in carbon enhanced growth of the organisms as compared to injection of organisms alone. Presumably the granuloma produced by the carbon provided a favorable medium for growth of the spirochetes. Chesney and Kemp (35) found that a non-specific inflammatory reaction induced by trauma or coal tar and involving the skin and subcutaneous tissue fostered the initiation and development of a primary syphilitic lesion in the rabbit. The most favorable time for inoculation of a wound was when it was granulating nicely and was well on its way to healing. The authors also described experiments by Levaditi and Yamanouchi (118) on growth of the spirochete in inflamed rabbit eyes and postulated that the inflammatory exudate furnished an increased food supply for the organisms. Chesney and Kemp, therefore assumed that the substances ("trephones" (29)) present which stimulate the growth of fixed connective tissue cells and the abundance of nutrients for new cell growth might also favor survival and growth of the *Treponema*. Such an hypothesis is in close agreement with the suggestion of Theobald Smith (183a) that the syphilis spirochete exerts a metabolic activity so close to that of the host that the latter reacts slowly and slightly to infection. Whether this means that the organism is intracellular at some time during its residence in the host or whether it may de-

rive its sustenance extracellularly remains to be determined. In either event, the close association of spirochetes and actively proliferating cells finds some confirmation in that the sites where the organisms are extremely numerous, such as the testicle of the rabbit and the organs of the human syphilitic fetus, are also centers of active host cell growth (35).

The rôle of the inflammatory reaction in enhancing infection by furnishing a site wherein the spirochetes settle preferentially from blood and lymph must also be considered (137).

It is interesting that *T. pallidum* despite its fastidious growth requirements will survive for months or possibly years in the animal host (66, 193). Perhaps this organism possesses the same faculty as the human tubercle bacillus (121) under adverse nutritional conditions of reducing its metabolism to a low level with the retention of respiratory and proliferative capacities.

*T. pallidum* rapidly succumbs to drying outside the body (66). Even drying from the frozen state thus far has been unsuccessful for preservation of these organisms (217), of *Leptospira* (189), and of *T. pertenue* (217).

#### E. Metabolism

Knowledge of the biochemical and metabolic characteristics of pathogenic spirochetes is very meager, particularly as providing a basis for identifying various species, distinguishing virulent from avirulent strains, developing synthetic mediums, or explaining factors in resistance. This may be attributed to the difficulty in cultivating and counting spirochetes and in obtaining sufficient numbers of them for metabolic studies. A few observations on spirochetal metabolism are summarized here. Further data, including a description of a partially successful synthetic medium for *Leptospira* (177), may be found in references (30, 31, 70, 163, 172, 176, 177, 195).

Noguchi (149) reported that addition of carbohydrates to the medium had no effect on the growth or morphology of *L. icterohaemorrhagiae*. Recently Chang (31) found that this organism did not utilize the simple sugars provided but probably subsisted on proteins or amino acids. He was unable to detect any consumption of oxygen by large numbers of leptospires during a period of several hours in the Warburg apparatus. Supniewski and Hano (195) observed that *Leptospira* utilizes L-arabinose but no other pentoses; it hydrolyzes galactose and glucose and to a smaller extent fructose and mannose. It breaks down urea but not uric or lactic acid. Scheff (196) made observations on *Borrelia recurrentis* and *Borrelia anserinum*. He found that when these organisms were grown in a medium containing glucose, they broke down the sugar to lactic acid without using up any of the oxygen. Fenyvessy and Scheff (54) made comparative studies of the metabolism of *Borrelia* and species of trypanosomes. They observed that *Borrelia* apparently did not use molecular oxygen and derived their chief energy from sugar fermentation, whereas trypanosomes used oxygen and obtained energy from oxidation of sugar.

There are some data in the literature on substances, usually vitamins in animal and microbial nutrition, which foster the growth of *L. icterohaemorrhagiae* and

*L. canicola*. Space is not available here for presentation of these data. However, it is of interest to note that many of the same vitamins which are growth-promoting factors for other microorganisms (ascorbic, nicotinic and pimelic acids, biotin, thiamine, calcium pantothenate, para-aminobenzoic acid, pyridoxine, nicotinic acid amide, riboflavin) also enhance the growth of *Leptospira* (31, 172, 176). These results indicate that enzyme systems similar to those in other microorganisms probably operate in *Leptospira*. Unfortunately, there are no comparable data on the spirochetes which are more difficult to cultivate *in vitro*.

#### F. Antigenicity and chemical composition

Early work indicated that serological cross-reactions could be obtained between spirochetes and trypanosomes but not between spirochetes and bacteria (46). In view of the significance of these findings in suggesting an antigenic relationship between spirochetes and trypanosomes, they should be confirmed by means of more accurate and refined modern immunological methods.

Spirochetes are generally considered to be gram negative in tinctorial properties (150, 208, 219). It has been suggested (208) that they stain poorly with methylene blue because of a lack of nucleoprotein in their protoplasm, but ability to stain with methylene blue is not definite evidence that an organism contains nucleic acid. Mudd (144) has found evidence that cultured spirochetes of the genus *Treponema* have surfaces whose wetting properties are suggestive of a high lipid content. Sensitization of these organisms with homologous immune serum results in an altered surface whose wetting properties are like those of protein.

*Leptospira*. Among spirochetes, *Leptospira* perhaps resembles the bacteria most closely in antigenic and possibly chemical properties. Following inoculation of killed or live organisms or during natural infection agglutinating, lytic, complement-fixing and protective antibodies are produced in high concentration (110, 219). These antibodies may persist in detectable titer for long periods of time, up to many years, in the blood of man and animals (110, 219). The immunity they engender is of correspondingly high quality and long duration (219). The antibodies are often highly specific, so that failure to obtain a demonstrable serological reaction against one antigen is not conclusive evidence of absence of infection with an organism containing another antigen (219).

There are different antigenic strains of *L. icterohaemorrhagiae* (66, 219). The specific antigens have not, however, been isolated. The only substance isolated in some reasonable degree of purity from spirochetes is a specific soluble carbohydrate from the non-pathogenic saprophytic *L. biflexa* (79). This carbohydrate, like the type-specific pneumococcal carbohydrate haptens, did not give rise to antibodies upon injection but was precipitated by rabbit antisera to the homologous strain, though not by antisera to pathogenic strains of *L. icterohaemorrhagiae* (79). Whether similar carbohydrate haptens exist in pathogenic *Leptospira* is unknown. The author (185) has immunized guinea pigs against fatal leptospirosis by repeated injections of cell-free supernatant from leptospiral cultures; this suggests that there is a soluble protective antigen in cultures of *L. icterohaemorrhagiae*. Hollande (83) has shown by the effect on tinctorial

properties after lipid extraction that there is lipid in cells of this organism. Carlinfanti (27) has presented serological evidence for the presence in *Leptospira* and in Wassermann beef-heart antigen of a common alcohol-soluble partial antigen. This is not surprising as lipid antigens of the Wassermann type have been found in a number of bacterial, plant and animal tissues (108, 226).

*Borrelia*. The pathogenic *Borrelia* induce the formation of lytic, agglutinating, complement-fixing and protective antibodies upon introduction into a suitable host (147). These antibodies are produced in high titer and apparently are closely connected with the mechanism of effective host resistance to infection.

There are no data available on the chemical composition of the relapsing fever spirochetes.

*Treponema*. The greatest chemical and antigenic non-conformist among the spirochetes is *T. pallidum*. This organism may unobtrusively enter the body causing little inflammatory reaction (55, 133, 137) and, in fact, may evoke no histological response by the host during its residence in the body during a period of months or years (34). Peculiar antibodies termed "reagins" are eventually produced by the host in response to infection (43, 208, 226). These "reagins" yield flocculation, precipitation, and complement-fixation reactions with spirochetal or tissue antigens. Avirulent culture strains of *T. pallidum* induce antibodies which give agglutination and spirocheticidal reactions *in vitro* with avirulent but not with virulent strains of the organism (239). There is growing evidence that protective antibodies are produced very slowly and in low concentration in the course of experimental or natural syphilis (18, 39, 212, 216).

*T. pallidum* and possibly other pathogenic treponemas (218) possess a Wassermann-type lipid hapten which is "exceptional among serologically active materials on account of its widespread distribution, surpassing in this respect even the Forssman antigens." (108). This ubiquitous hapten is found in proteus bacilli, *Leptospira*, trypanosomes, tubercle bacilli, plants, milk, egg yolk, and in normal tissues of man and animals<sup>5</sup>. Bergel (9) has shown that *T. pallidum* contains a great deal of lipid in the cell membrane. He believes (9) that chemotactically this lipid produces a lymphocytic reaction which in turn, via lipolysis, leads to disintegration of the spirochetes. He considers the "reagins" or anti-lipoids as products of lipolytic lymphocytes which appear as reaction against the lipid antigens.

That there are heterologous antigenic strains of *T. pallidum* is suggested by the relatively specific character of the resistance of rabbits to reinfection (38). The resistance against the homologous strain of organism originally employed for inoculation usually is greater than against heterologous strains (38).

Turner and his co-workers (218), by cross immunity studies of experimental syphilis, yaws, and venereal spirochetosis of rabbits, have found evidence to indicate that the treponemas causing these infections are biologically and antigenically related. Rabbits infected by intratesticular injection of *T. pallidum*, or

<sup>5</sup> For further information on the Wassermann antigen and its relationship to biologic false positive serologic reactions in syphilis, the reader is referred to two recent reviews (43, 226).

*T. pertenuis*, or *T. cuniculi* developed within 6 months a substantial amount of cross immunity to challenge intracutaneous injection of each of the same species of spirochetes.

#### G. Toxins and aggressive substances

Sanarelli and Pergher (175) believe that spirochetes do not themselves produce pathological changes but only render the host more susceptible to damage by secondary invaders. The observations of Zavagli (234) that dead cultures of *L. icterohaemorrhagiae* increase the severity of *Trypanosoma brucei* infection in mice while dead bacteria had no effect support the supposition of Sanarelli and Pergher. However, Taylor and Goyle (204) and Stavitsky (185) cultured organs of guinea pigs experimentally infected with leptospiras and isolated only the spirochetes, except when the organs were cultured many hours after death and were consequently contaminated with intestinal bacteria. When paratyphoid bacilli or streptococci were introduced into guinea pigs approximately at the height of leptospiral infection, these organisms either did not affect the course of the spirochetosis, or the new infection supplanted the old, only the bacilli or cocci being present in the organs at necropsy (185).

*Leptospira*. Pettit (157) gave guinea pigs up to 10 ml. of filtrates of cultures of *L. icterohaemorrhagiae* with no ill effects. Fukushima and Hosoya (61) found substances toxic for guinea pigs in cultures of these spirochetes which, having been maintained under anaerobic conditions, contained dead and dissolved organisms. Higuchi (76) also demonstrated a toxin in pure cultures of leptospiras which had been kept at 37 C *in vacuo* for 2 to 3 days. Injection of this toxic material into guinea pigs produced an increase in the bilirubin of the blood, hyperemia of the bulbar conjunctiva, and fever (76). However, attempts to confirm these studies were negative (186). Further efforts to demonstrate toxin in various cultures or tissue extracts from infected animals were unequivocally negative. Stavitsky's experiments (186), although not excluding the existence of a spirochetal toxin, make it unlikely that it is a potent exotoxin such as is produced by *Corynebacterium diphtheriae*, or a stable endotoxin such as can be extracted from cultures of *Vibrio comma*. The toxin or toxic antigen of *L. icterohaemorrhagiae* may be extremely labile and rapidly destroyed in the presence of the chemical and physical agents used in preparing the test spirochetal extracts.

Attempts to demonstrate hyaluronidase (50), fibrinolysin, leukocidin or coagulase in leptospiral extracts were negative (185). As far as is known no attempts have been made to demonstrate these enzymes in other spirochetes.

*Borrelia*. No clear-cut demonstration of a toxin from borrelias has been found in the literature.

*Treponema pallidum*. Brown and Pearce (19) consider the possibility of a toxin as a factor which is distinct from the spirochetes in exciting infection. They believe certain features of the syphilitic reaction may find their explanation in the action of a toxin. However, Kolmer (104) could not demonstrate filtrable or soluble exogenous toxins in cultures of spirochetes alleged to be *T. pallidum* but avirulent for rabbits, or in filtrates of the tissues of acute testicular

syphilis in rabbits. Feebly toxic substances, possibly endotoxins, were obtained from cultures of spirochetes subjected to autolysis or desiccation, grinding and extraction (104).

### H. Tropisms

1. Chemotropism (chemotaxis). The apparent lack of chemotropic properties in pathogenic spirochetes is discussed in section II D.

2. Histo- and organo-tropisms. The concept of selective affinity of infectious agents for specific organs, tissues or even cells where they find conditions favorable for survival and proliferation has been strengthened considerably by recent studies on these tropisms of viruses (224).

*Leptospira*. *L. icterohaemorrhagiae* appears to have an affinity for the liver in experimental infection of the guinea pig. Within 7 hours after injection into this animal the organisms may be recovered from the liver (25). The extensive damage done to the liver in leptospirosis (219) is further evidence of some special suitability of this organ for multiplication of the spirochetes. In this connection, the finding that virulence of *Leptospira* may be maintained *in vitro* if a small amount of emulsion of fresh guinea pig liver is added to the medium (30) is interesting. Experimental and clinical observations have suggested that strains of this organism may localize in the meninges in the course of infection (158, 210). However, attempts to develop a "meningotropic" strain of *L. icterohaemorrhagiae* by repeated subdural passage in guinea pigs were unsuccessful (186). Other affinities of these organisms for the eye (186, 187, 219), adrenals (186, 219), bone marrow (47, 186), and kidney (219) require further study.

*Borrelia*. There is considerable material in the literature on latent infection of the brain of experimental animals with these spirochetes (26, 143). Whether this represents a true tropism is not known. It is known that certain species of these organisms may pass through the blood-brain barrier (26, 60).

*Treponema*. There has been much research on the question of neurotropic strains of *T. pallidum* (114, 193). This work has given rise to the postulate (193) that prolonged residence in a particular tissue induces permanent changes in this organism. Thus, sojourn in the mouse brain may prepare the spirochete for the development of a neurotropic reaction in the rabbit (193). Presumably the pronounced neurotropic tendencies often manifested by *T. pallidum* in man (114, 193) are attributable to infection with or development of a neurotropic strain of the organism. *T. pertenue*, causative agent of yaws, is often considered a variant of *T. pallidum* which, by residence in the negro, has developed dermatropic affinities (155). Bessemans (11) found that low temperature in tissues favors the multiplication of *T. pallidum* in experimental syphilis. The organisms apparently settle in those tissues which have lower temperatures, such as the testis.

### I. Spirochetal variation

This is certainly one of the most important phases of spirochetal biology as it influences all aspects of spirochetal behavior and, therefore, the pathogenesis and resistance in these diseases. Braun (13) and Luria (126) have discussed various

phases of bacterial dissociation or variation. These discussions have proved useful for providing at least a tentative understanding of the possible mechanisms of spirochetal variation. In the absence of significant information on spirochetes, therefore, we shall have to rely upon reasoning by analogy, always a hazardous procedure, in the attempt to understand spirochetal antigenic variation.

1. Antigenic variation. The data on antigenic variation among spirochetes are most complete for the borrelias (143, 147, 180). The generally accepted facts of the phenomenon of antigenic variation of borrelias and the apparently related clinical relapses are presented in Section II G. Whether these antigenic variations are spontaneous, that is occurring continuously at a certain rate regardless of whether the organisms are in contact with specific antibodies or substrate, or are induced by contact of organisms with specific antibodies or substrate, is not known. In the first case the antibody acts merely to select the new variant, permitting it to outgrow the original normal form. In the second case the antibody itself induces the variation resulting in adaptation. In analogous cases in bacteria the new variant has appeared independently of the substrate to which it was adapted. Resistance to penicillin of *Staphylococcus aureus* (44), resistance to phage of *Escherichia coli* (125), and the requirement for uracil by clostridia (174) have been found to occur spontaneously, independently of the environmental agent.

2. Variations in virulence. There are strains of spirochetes of different degrees of virulence (63, 143, 219). As with other microorganisms, virulence is gradually lost upon cultivation *in vitro* and often regained upon animal passage. An exception is the maintenance of virulence by *L. icterohaemorrhagiae* *in vitro* if a small amount of emulsion of fresh guinea pig liver is added to the medium (30). The basis for the variation in virulence is obscure. Many factors, such as antigenic variation, variation in chemical composition, and possibly variations in the magnitude or sign of the electrokinetic potential may be involved. The virulence of the organisms may influence the development of the carrier state or relapses by the host. If the organisms are virulent, they may stimulate the defensive responses of the host and conduce to a more solid immunity or else overwhelm the host's defenses and kill the animal (97). Thus mildly virulent strains of *Leptospira* lead to relapses (5), whereas virulent strains rarely do.

An arresting observation is that syphilitic organisms from rapidly growing chancres in rabbits tend to give rise to more rapidly growing chancres with a shorter incubation period in normal rabbits than do spirochetes from slowly growing lesions (18). It is not known whether this effect depends on the alteration of the organisms' potential for proliferation *in vivo* or on the carry-over of nutrients or protective colloids with the chancre material.

Turner and his associates (218) believe that stability rather than variability is generally the rule with regard to pathogenic properties of treponemas. If variants do occur, either the rate at which they appear is small, or conditions for their survival are unfavorable.

3. Variations in susceptibility to drugs and antibodies. These variations are



known to occur in spirochetes and to persist through succeeding generations of the organisms (97, 193, 219). Basically they probably are related to variations in antigenicity or chemical structure (including enzyme systems), so that drugs or antibodies cannot be adsorbed at certain of the "active centers" (162) of the organisms and, therefore, cannot accomplish their effects.

## II. HOST FACTORS THAT MAY INFLUENCE HOST RESISTANCE

### A. *Natural resistance*

The separation between natural and acquired resistance is artificial and arbitrary, as these properties are obviously interdependent. This point is particularly well clarified in Rich's recent monograph on the pathogenesis of tuberculosis (168). For example, the apparent greater resistance to an infection of older compared to younger individuals may be simply the consequence of prompter and greater antibody production by the elder animals (6). On the other hand, the ability of the rabbit by raising its body temperature to resist infection with pneumococcus III (170) seems to be an unquestionable example of natural resistance. It is, therefore, imperative that the basic mechanisms involved be understood before native and acquired resistance can be separated rationally.

*Leptospirosis.* Apparently there is no complete natural resistance to leptospirosis in man and animals (219, 225). Sex differences in resistance have not been demonstrated. An increase in resistance with increased age has been shown in the guinea pig (185, 192), white mouse (109), dog (219, 232), and rabbit (219). Species resistance to the disease is generally recognized in the rat, mouse, rabbit and dog (219, 225). Symptomless infection is particularly common in these species. There are also differences in resistance of various genera of mice of which the deer mouse (*Peromyscus*) is most susceptible (154). Within various species, moreover, there appear to be strain differences in resistance which have been most clearly defined among strains of white laboratory mice which vary from extreme susceptibility (109) to great resistance (71, 188). The guinea pig and hamster are the species of laboratory animals most susceptible to leptospiral infection (113, 186). There are individual differences in susceptibility to this infection among animals of the same species (185, 219). However, it is difficult to exclude inapparent or past infection and the consequent production of some active immunity in these individuals. Moreover, few strains of laboratory animals are genetically homozygous, and it is probable that these individual differences in response to infection are merely expressions of genetic strain differences in the animals.

There have been a few studies of the mechanisms of age and species resistance in leptospirosis. Some of the data are included in the following sections B, C, and D. Here only the most recent data are given.

Higuchi (75) attributed the great resistance of rats to fatal leptospirosis to strong antibody formation and destruction of organisms by lytic antibodies. He found that rats were easily infected, but their symptoms were light. Stavitsky (187) studied peripheral barriers, non-specific inflammation, normal and immune whole blood and serum, cell-free inflammatory fluid and phagocytic cells

in old and young, normal and immune animals of resistant and susceptible species which were injected with *Leptospira* (187). In all groups only lytic antibodies exerted a significant effect on the organisms *in vitro* or *in vivo*. The organisms did not appear to damage the tissues of rats and mice as much as those of guinea pigs and hamsters (185). Although these strains of rats and mice rarely succumbed to the infection, the ease of invasion and spread of the spirochetes in these hosts, the findings of hemorrhagic pulmonary lesions, and the development of a carrier state (185, 186, 187, 219) indicated that resistance of these species to infection is relative rather than absolute. In view of the importance of antibodies in resistance to this disease, it was suggested (187) that larger numbers of animals be studied to determine the comparative ability of young and old animals and susceptible and resistant species to form antibodies against the spirochete.

*Relapsing fever.* Relapsing fever apparently is primarily a disease of lower animals; its occurrence in man is accidental (143, 147). The disease does not seem to make distinctions as to age, sex, color or social status of man (143, 208). All animals except apes, rabbits, guinea pigs, mice, and rats are considered naturally resistant (143, 147, 208).

There are some data on the relationship between age and resistance to relapsing fever (147, 156). In 95% of old mice inoculated with a strain of *Borrelia*, organisms were in the blood in 3 to 10 days, while of young animals 64% showed no infection, 24% light infection and 12% medium infection (147a). In the younger animals the infections were never serious or fatal and the period of incubation was 1.8 days shorter. On the other hand, an increased resistance to *Borrelia* of guinea pigs with increasing age has been shown (69). Interesting studies have been made of the comparative susceptibility of the chick embryo, the chick, and the adult bird to infection with *Borrelia duttoni* (152). The embryo was susceptible but the newly hatched chick and adult bird were not. Some inoculated embryos harbored organisms after hatching but these were disposed of by the rapidly developing defensive mechanism of the hatched bird. The circulating blood was eliminated as a basis for the natural immunity of the chick when phagocytosis and spirocheticidal effects of serum and by cells of whole blood of embryo or chick could not be shown.

There are species differences in response to relapsing fever spirochetes although relapses are common in many species (78, 143, 209). However little is known of the basis for these differences.

*Syphilis.* Syphilis is naturally acquired only by man (34). The hypothesis of early South American explorers that syphilis existed in llamas there and that infection was originally acquired by natives from these animals has not been substantiated (34). South American workers (87) reported the successful reproduction in llamas of all of the essential features of human syphilis, but Zinsser (236) states that this work has never been supported by subsequent investigation. Monkeys and rabbits can be infected and the organism transmitted indefinitely in them (34). Other species are more or less resistant to frank infection (34). Rats and mice, for instance, invariably develop an asymptomatic infection in which the spirochetes invade the tissues without the production of lesions or

clinical signs of illness (66, 208). Most other species, though occasionally reported to have been infected, usually manifest complete resistance to syphilis (34). Experimental infection in monkeys and man corresponds very closely to natural human infection (208). In rabbits many of the lesions seen in human infection are often duplicated (208). The organisms may remain latent in the lymph nodes (22) or in one or more of the internal organs (184). A positive Wassermann reaction is usually produced (34). Late lesions are not produced in the monkey and rabbit (34).

Rosahn (171) has presented evidence for breed, race or family resistance to syphilis in rabbits. In studies of the reaction of standard breeds of rabbits to experimental syphilis, he found that Havana and Dutch animals were relatively resistant whereas English, Himalayan and Rex rabbits were relatively susceptible. Frazier and Mu (57) found albino rabbits less resistant than brown rabbits. Brown and Pearce (20) obtained poor results with scrotal inoculation of Belgian and Flemish giants while small albinos, grays, browns and Dutch belts gave satisfactory results. In general the "higher" apes such as the chimpanzee react more like man to infection (34, 208) whereas in "lower" apes like macaques the disease is less easily produced and on the whole milder (34).

Age appears to be a factor in experimental rabbit syphilis (33). Inoculation apparently produces lesions more often in younger rabbits (33, 34, 184). Sexual differences in susceptibility to syphilis are particularly outstanding in man and animals (156). The female exhibits a markedly greater resistance than the male (156). Unquestionably, this exalted resistance is somehow related to the effects of the female sex hormones, the estrogens, as will be elaborated in a later section.

There appear to be no naturally immune individuals among man or susceptible species of animals, although there are individual human, monkey, and rabbit reactions to infection.

Although the mechanisms of natural resistance to *T. pallidum* have not been studied extensively, there are suggestive data on the influence of physical constitution of rabbits on their resistance (23). In these experiments rabbits were subjected to varying forms of light. It was found that the light increased resistance to infection in proportion as it affected the physical constitution of normal rabbits, and the organs most affected were those concerned in the animal's reaction to syphilitic infection. Zinsser and Hopkins (237), studying the mechanism of natural resistance of mice against *T. pallidum*, could not detect phagocytosis of the organism in the peritoneal cavity of this species. They concluded, therefore, that phagocytosis is not primarily responsible for the marked resistance of this host.

### *B. Peripheral barriers of portals to infection*

The first reaction of the host is usually an attempt to localize the infective agent at the site of entrance into the body (166). It is therefore of interest to determine how effectively spirochetes are localized at the various portals of entry to the body.

All of the pathogenic spirochetes appear to invade the body rapidly and un-

obtrusively by most portals of entry (85, 147, 150, 208, 219). This may in part be due to their rapid motility which is most readily observed *in vitro*. The apparent boring of the avian spirochete into macrophages in tissue culture (77) supports this suggestion. *T. pallidum* has been known to bore through a solid medium, and *Borrelia recurrentis* through a semi-solid one (150). The boring of *Leptospira* through agar has been compared aptly to 'the gyring and gimbling of Lewis Carroll's slithy toves.'

*Leptospira*. In the guinea pig and hamster *Leptospira* invades the body quickly after injection by peripheral routes including cutaneous, meningeal, intraperitoneal, intraanal, intravaginal, conjunctival and intraocular (85, 186, 187, 221). They may enter the human body through intact skin (85, 150). Schüffner (178) has taken advantage of the great invasiveness of these organisms to devise a method for separating them from contaminants. The contaminated spirochetal suspension is inoculated intraperitoneally into a guinea pig and 10 minutes later the animal is bled from the heart. Invariably the spirochetes may be isolated from this blood. It also has been shown by extirpation of the injection site (186) that 5 minutes after intradermal injection of these organisms into guinea pigs, enough have left the area of injection to cause death of the animal. Only intact skin and nasal mucous membrane seemed to afford an effective barrier to invasion of the body of the guinea pig (187). The oral route was not as effective as the other peripheral routes (187), probably due to destruction of the organisms by gastric juice (208).

Since "every intradermal injection is truly intralymphatic" (136) the spirochetes probably escaped from the site of intradermal injection through the superficial lymphatics. Attempts to demonstrate fibrinolysins and "spreading factor" (50) in filtrates and autolysates of leptospiral cultures were unsuccessful (185). It was, therefore, suggested (186, 187) that the great speed with which the organisms revolve in cork-screw fashion enables them to bore through connective tissue as well as fibrinous networks and other localizing barriers erected by the host (185, 186, 187). The organisms were not localized at the site of intradermal inoculation (186, 187). Nor could they be isolated from regional lymph nodes after peripheral injection (186, 187), suggesting that they pass rapidly through these structures into the blood, few of them being trapped in the nodes. The eye is a particularly favorable portal of entry to the body as the organisms appear to multiply abundantly there (186).

When injected into the dermis, leptospiras produced a very mild inflammatory reaction there during the first 24 hours after injection as indicated by histological studies and by the free diffusion to the tributary lymphatics of trypan blue (137) inoculated into the area (187). When injected intraperitoneally, intraocularly and intrameningeally, they produced progressively more intense inflammatory reaction and correspondingly more localization of spirochetes in that order (187). However, it is not known which is primary, rapid escape of the organisms from the injection site before causing much local cellular injury or their non-irritant quality *per se*. It is possibly significant that the organisms escaped from a peritoneal cavity which was artificially inflamed so that intraperitoneally

injected streptococci, horse serum and trypan blue were unable to spread through the regional lymphatics (185). *In vitro*, the spirochetes wriggled through rather dense networks of fibrin which trapped many other species of motile microorganisms (188). Perhaps a very closely-knit fibrin coagulum and complete lymphatic blockade are required to restrain their spread (137).

After inoculation into the brain or meninges, *L. icterohaemorrhagiae* often can be isolated from the blood; and occasionally they have produced generalized leptospirosis in guinea pigs and red foxes (185). However, despite positive blood cultures, these spirochetes do not readily, if ever, reach the brain or spinal fluid through the blood vessels, as judged from negative brain and meningeal cultures (185). This paradoxical situation is, nevertheless, only apparent. The walls of the meningeal blood vessels provide an impenetrable barrier for foreign particles which gain entrance into the general circulation (132). The passage of substances from blood to spinal fluid is mediated by the choroid plexus. This structure, unless damaged, is impermeable to colloidal substances (59). On the other hand, it can be shown with the aid of aniline dyes that in small animals intracranially injected material always reaches the lateral ventricles (59). The passage of substances from the ventricles to blood takes place through the venous sinuses which are permeable even to corpuscular elements (59). It is quite understandable, therefore, that spirochetes go from spinal fluid to the blood but not in the opposite direction.

The negative brain cultures in guinea pigs after peripheral injection of leptospiras are in keeping with a concept of Friedemann (60). This worker has explained the impermeability of the capillaries of the central nervous system (blood-brain barrier) to aniline dyes, toxins, viruses, antibodies and drugs on the basis of the electrochemical properties of these substances. This barrier appears to be permeable to substances carrying a positive or no surface potential while it is impermeable to those bearing a negative charge at the pH of blood. He feels that this permeability possibly may be correlated more correctly with the magnitude rather than the sign of the zeta potential. However, as previously summarized (Section I B) there is no agreement on the surface potential of *Leptospira*. Therefore, careful cataphoretic studies are required to establish the zeta potential of leptospiras under various conditions. In that way it may be decided whether the impermeability of the cerebral blood vessels to the spirochetes is correlated with the electrokinetic potential of the organisms. It may be that different species of animals vary in the permeability of their blood-brain barrier to leptospiras. Also, the spirochetes may be able to change the sign of their zeta potential as some trypanosomes do (16).

In view of these facts, investigations of the correlations between electrokinetic potential and ability to invade the central nervous system would be of great interest. The oft-discussed problem of neurotropic strains of the syphilis and other spirochetes may thus be related to the surface potential of the organisms.

*Borrelia*. These also are rapidly invasive, apparently through the unbroken skin (147). Twenty-four hours after intraperitoneal inoculation into mice, the organisms may be demonstrated in the blood. There are no data on their local-

ization in the skin or in regional lymph nodes. Schuhardt (179) has seen no evidence of a prompt and severe inflammatory response to artificially injected *Borrelia*.

*Treponema pallidum*. This organism invades the body rapidly after peripheral inoculation. It can pass through apparently intact skin and mucous membrane (35), though it is impossible to exclude the presence of minute breaks in these tissues (150). The organisms also can pass through granulating wounds (35). Trauma and abrasion favor invasion (35, 36) but apparently are not always necessary. There is evidence of invasion of the body through the lymphatics and blood since there is an inflammatory reaction in the perivascular lymphatics (193, 208). The organisms settle out in inflammatory areas and produce lesions there when inoculated intraperitoneally or intratesticularly (36).

Kolle and Evers (103) infected rabbits by cutaneous or subcutaneous inoculation into the scrotum, removed the inguinal lymph nodes after varying periods, and injected these into fresh animals. By this means they found that the nodes were infective within 30 minutes of the injection. They were able to show that in guinea pigs the spirochetes reached the focal lymph nodes 5 minutes after cutaneous inoculation of the scrotum. In apes the time between inoculation and invasion is probably longer. For example, a chimpanzee anointed locally with calomel ointment 1 to 2 hours after cutaneous inoculation never developed syphilis (138). During the primary stages organisms are found in the local chancre and sometimes can be demonstrated in the blood (193). Mahoney (130) deposited a suspension of *T. pallidum* on intact genital mucosa of male rabbits, and at successive intervals killed animals for histologic study. One hour after exposure began the organisms occupied a more or less protected position in the crypts of the mucous membranes. In 2 hours there was evidence of penetration of the deeper tissues, and in 3 hours the organisms had penetrated to a depth that would preclude direct influence of any chemotherapeutic agent applied to the surface. *T. pallidum* apparently can traverse the blood-brain barrier and pass from blood into the brain substance (60).

### C. Humoral mechanisms\*

1. Normal blood. Taylor and Goyle (204) reported that freshly drawn human blood had spirocheticidal properties for *L. icterohaemorrhagiae* *in vitro*. There was no study of the effect of blood on the organisms *in vivo*. Stavitsky (187) was unable to detect any spirochetostatic, lytic, or agglutinating effect on the spirochete *in vitro* of normal defibrinated whole blood from adult man, rhesus monkeys, dogs, foxes, guinea pigs, rabbits, hamsters, white mice, and white rats. Oag (152) observed that mouse, chick embryo and chick bloods were spirocheticidal *in vitro* for *Borrelia duttoni*, possibly by a direct lytic effect. However, such a property apparently was absent from the blood of mouse and chick embryo *in vivo*. Turner and Diseker (215) demonstrated that *T. pallidum* loses its virulence and, probably, infectivity after 72 hours' storage in citrated human or rabbit blood at 2 to 4 C; and Bloch (12) found that *T. pallidum*, stored in citrated

\* Antibodies are discussed under Acquired resistance, Section II E.

blood at 5 C, retained its virulence for 3 but not 4 days. He stated that the presence of tissue may favor the maintenance *in vitro* of virulence of the organisms.

2. Normal serum or plasma. Marked differences exist in the ability of serums derived from different species to support the growth of *Leptospira in vitro*; rabbit and horse serums favor growth while most other serums support poorer or no growth (186, 219). However, no correlation may be made between the ability of their serums to support growth and species susceptibility to *Leptospira* (186, 219). Normal dog serum has no protective power against this organism (219). Normal serum from man, horse, goat, rabbit, guinea pig, white rat, hen, and sheep contain no agglutinins for *Leptospira* (221). According to Corrales (40), the serum of horses, lower apes, rabbits, guinea pigs, or mice does not exert a spirochetolytic effect *in vitro*. Hen and eel serums were found feebly spirochetolytic (40). Normal serums from man, monkeys (*Macacus rhesus*), dogs, foxes, guinea pigs, rabbits, hamsters, white mice, and white rats were without demonstrable effect on the morphology or growth of the spirochetes *in vitro* (187). Turner, Bauer, and Kluth (217) reported that *T. pallidum* and *T. pertenuis* suspended in blood serum were killed during freezing in carbon dioxide and alcohol and desiccation. Selbie (182) found that *T. pallidum* kept in rabbit plasma at 5 C maintained its virulence for 6 but not for 10 or 21 days. Ravitch and Chambers (162a) showed that *T. pallidum* and relapsing fever spirochetes lost their virulence gradually over a period of days or weeks during storage in frozen plasma at -12 and -20 C. Probey (161) observed that *T. pallidum* suspended in saline-normal horse serum is apparently killed during the deep freezing and drying process since material restored immediately after processing was not infective for rabbits.

3. Bile and bile salts destroy *L. icterohaemorrhagiae* rapidly *in vitro* (150). Taylor and Goyle (204) believe that the bile which is formed as a result of destruction of blood cells or through some other mechanism in leptospirosis is at least partly responsible for the disappearance of the organisms from the blood. According to Noguchi (150), *T. pallidum* is disintegrated by 10% bile salts. There are no data on the effect of bile on *Borrelia*.

4. Human gastric juice destroys *Leptospira* in 30 minutes (208). No data were found on the effect of gastric juice on *Borrelia* and *T. pallidum*, but it might be expected that they too would be destroyed in this highly acid medium.

5. Inflammatory fluid. Corrales (40) reported that rat, rabbit, and mouse inflammatory fluid devoid of cells was spirocheticidal for leptospires *in vitro*. The organisms became deformed and granular, their mobility decreased and finally they disappeared. However, Stavitsky (187), using cell-free inflammatory fluid from guinea pigs, mice, and rats could not confirm these observations. Nor could he discern any effect of a peritoneal exudate on *Leptospira in vivo* (187). There are no data on the effect of inflammatory fluid on the other pathogenic spirochetes.

6. Hormones. There has been a great deal of research on the influence of various hormones on syphilis (156). Women react to syphilitic infection as

though a different species from man; they are so much more refractory to this infection (193). There is little doubt that pregnancy is an important factor in altering the course of syphilis (95, 156, 193); it may be responsible for the comparative freedom of women from neurosyphilis (193). Infections in a group of male and non-pregnant female rabbits were more severe than in a group of females in which impregnation and infection occurred at about the same time (17). Lowered resistance to syphilitic infection was observed in ovariectomized rabbits (84).

A hormonal basis for these phenomena has been made very plausible by studies in experimental syphilis and tuberculosis. Estrogens given to male and female rabbits appear to inhibit the syphilitic infectious process (58, 95). The most striking modification of response was the resistance to the disease developed in the testis (58). Lurie (128) has found that estrogens also exert a profound inhibitory effect on experimental tuberculosis in the rabbit.

Experimental syphilis of the rabbit has been reported to be more severe in animals after complete thyroidectomy than in controls (159). Partial thyroidectomy resulted in a milder disease than in control animals. Complete thy-mectomy produced a less pronounced effect than either partial or complete thyroidectomy but, in general, the syphilis resembled that in partially thyroid-ectomized animals (159).

It seems, therefore, that in syphilis the integrity of the endocrine glands and balance of endocrine functions are valuable adjuncts in defense of the host.

#### *D. Cellular mechanisms*

No feature of anti-spirochetal resistance is so controversial as the cellular mechanisms. In part this is due to incomplete knowledge of the diverse ways in which cells may take part in host defensive reactions. The all-important question of cells in defense through their rôle in antibody formation will not be considered as it is an extremely broad and disputatious subject in itself. The reader is referred instead to a recent paper (53) which discusses cells in connection with antibody production. Here attention is focussed mainly on the thorny question of phagocytosis as a defensive weapon in spirochetal infections.

It is very difficult to determine whether or not phagocytosis of spirochetes occurs either *in vitro* or *in vivo*. Many spirochetes are lysed extracellularly and possibly intracellularly upon contact with specific antibodies, and may leave within phagocytic cells no clearly recognized vestige which may be distinguished from normal intracellular granules. It is very difficult to recognize a spirochete within a cell by means of the dark-field technic. Moreover, one has to distinguish between adherence of the organisms to the leukocytes and actual ingestion, also between the active boring of the organisms into cells (77) and their passive ingestion. It is often not possible to determine whether a spirochete is within a cell or above or below it in wet or fixed preparations. However, one might expect in the course of numerous dark-field examinations to witness the actual engulfing of the organisms, if the process occurs at all frequently.

The great length of some strains of pathogenic spirochetes (4 to 30  $\mu$ ) 98, 208,



219) might preclude their ready phagocytosis even by large macrophages without: *a.* lysis or curling of the organisms; *b.* agglomeration and fusion of phagocytic cells (foreign-body giant cells) about the spirochetes. Phagocytosis of spirochetes accompanying their lysis has been reported (97, 101, 106, 107, 151, 219). The presence of coiled intracellular organisms likewise has been noted (117). Phagocytosis of spirochetes by foreign body giant cells has not been described. Partial ingestion of spirochetes may be seen in illustrations to an article by Kritschewski and Sinjuschima (106).

The active motility of spirochetes may render phagocytosis difficult in the absence of some immobilizing agent such as a specific agglutinating or lysing serum (233). However, Himmelweit (77) has described the apparent active boring of the avian spirochete into macrophages giving the appearance of phagocytosis.

Teale (205) has emphasized the importance to the phagocytic process of sedimentation of fibrin meshworks with subsequent trapping of the organisms in them. It may be significant, therefore, that leptospiras, probably due to their motility, do not sediment readily. Approximately 5 hours' centrifugation at 3000 rpm was required to clump them completely from a suspension (185).

There has been no definite demonstration to date that the pathogenic spirochetes are positively chemotactic. *L. icterohaemorrhagiae* did not attract phagocytic cells *in vitro* or *in vivo* despite the coexistence in one tube or in a preformed inflammatory exudate of large numbers of spirochetes and phagocytes (185, 187). There was no evidence that the spirochetes produced leukocidin, (208 because the phagocytes engulfed cocci introduced into the *in vitro* mixture (187). Novy and Knapp (151) considered as chemotactic the distorted *Borrelia* in rats which have recovered from relapsing fever. However, it is questionable whether this term may be applied to organisms sensitized and degenerated by contact with specific antibodies. McCutcheon (134), in a review of chemotropism, states that specific antibodies appear to play no part in chemotaxis which is a non-specific phenomenon often displayed by antigens. Bergel (9) believes that the lipid cell membrane of *T. pallidum* chemotactically induces a lymphocytic reaction, but McCutcheon (134) states that lymphocytes do not exhibit chemotaxis to any substance yet studied. He knows of no evidence that *Leptospira* or *Treponema* causes a chemotropic response and considers it unlikely that they do (133).

There are at least three possible explanations for the apparent lack of chemotropic properties in pathogenic spirochetes. First, the organisms may not produce substances in the course of their metabolism that attract phagocytes (134). Second, they may not injure tissues, which may then release chemotactic substances (134). Third, the organisms may escape from the focus of infection without having released chemotropic substances. Under such conditions the tissues may also fail to produce these agents.

*Leptospirosis.* Only the humoral aspects of host resistance in leptospirosis have been given much attention (187, 219). The rôle of the fixed and mobile phagocytes has been almost entirely neglected except insofar as investigators

have studied these cells in stained smears or sections of tissues from naturally or experimentally infected men and animals (219).

Inada *et al.* (85) found spirochetes in epithelial and phagocytic cells in man. Degenerated organisms were also observed in lymph nodes and spleen. The occurrence of intracellular spirochetes was attributed to the spirochetes' invading the cells in order to escape the action of the antibodies; while the presence of spirochetes in epithelial cells of glands perhaps indicated their way of escape from the body. Vanni (223a) observed that leukocytes between liver cells and in blood vessels were filled with nests of spirochetes in which individual organisms were still recognizable. Corrales (40) has described phagocytosis of leptospiras by phagocytic cells in the peritoneal cavities of mice, rats, guinea pigs, and rabbits following intraperitoneal injection of the organisms. He concluded that natural resistance of such species as mouse, rat, and rabbit is due to phagocytosis, destruction of spirochetes by substances elaborated by the leukocytes, and the inability of the organisms to injure the tissue cells of these species. Hindle (78) states that phagocytosis of the spirochetes is extremely active in monkeys, rabbits, rats and mice, both *in vitro* and *in vivo*, but presents no experimental basis for the statement.

In view of the significant nature of Corrales' work (40), Stavitsky (187) attempted to confirm and extend his studies. However, not a single instance of phagocytosis of *Leptospira* was observed in the course of numerous examinations, *a*, of exudates from infected eyes, peritoneal cavities, or blood containing spirochetes, *b*, of *in vitro* mixtures of serum (normal and immune), polymorphonuclear and mononuclear leukocytes (from normal and immune animals), and spirochetes, and *c*, of stained smears and sections of brain, meninges, liver, spleen, adrenals, bone marrow and kidneys of infected animals. The organisms were agglutinated or lysed only in the presence of specific antibodies. Mononuclear cells in cell cultures were without effect on the organisms. When *Leptospira* were introduced into a skin area containing a preformed predominantly polymorphonuclear inflammatory exudate, no localization or phagocytosis of the spirochetes in the injection site was observed (185).

Tscherikower and Rubinstein (211) found no significant difference in the response to leptospirosis of splenectomized and normal guinea pigs, and concluded that there is only a slight protective function of the reticulo-endothelial system in this disease. In another study (233) in which the reticulo-endothelial system of guinea pigs was blocked by intracardial injection of ferric sucrose 2 days after splenectomy, the surviving animals behaved much as did the normal ones as regards active and passive immunization and response to bismuth therapy. Splenectomy in mice did not alter the course of subsequently introduced infection (185). However, these experiments are open to the criticism that blockade and splenectomy may not have lowered the functional activity of the reticulo-endothelial system sufficiently to produce a noticeable effect.

In contradistinction to most microorganisms (205, 208), *L. icterohemorrhagiae* apparently was not cleared by cells of the reticulo-endothelial system from the blood of normal guinea pigs, mice, and rats during the first 48 hours after intra-

cardial injection; there was no appreciable fluctuation in the number of organisms in the blood during this time (187). In other experiments (185), organisms were cultured from the blood up to 120 hours after injection. Chang (30) has reported that avirulent leptospiras may be found in the blood of guinea pigs for a week or more after inoculation.

Benzene administered to produce a profound artificial leukopenia in mice was without detectable effect on leptospiral infection in naturally resistant strains of white mice (188).

*Relapsing fever.* In no field of immunology has the question of cellular versus the humoral basis of immunity been asked so persistently as in relapsing fever studies (143, 147). Some have considered resistance as due to phagocytosis, and others to lytic action of specific antibodies with phagocytosis a secondary phenomenon (66, 143, 147). It is well, therefore, to review the evidence on the question.

It is generally agreed that *Borrelia* are usually seen extracellularly in tissue sections (97, 143, 147). Many intracellular fragments or granular corpuscles may be observed occasionally, but there is no conclusive evidence that these are related to the spirochetes (97, 147). Dark-field examination may show spirochetes entangled in the pseudopodia of polymorphonuclear leukocytes, but there is little to suggest that ingestion ever occurs (97). Fixed preparations do not reveal phagocytosis by wandering phagocytes (97). Spirochetes are invariably extracellular in blood films (97). Histological sections of infected tissue likewise give no indication of phagocytic action by mobile leukocytes (97). Oag (152) did not observe phagocytosis of *Borrelia duttoni* by any circulating cell in the blood of chick embryo, chick, or mouse. Other investigators have reported phagocytosis of *Borrelia* by wandering cells in blood and tissue (66, 143, 147). However, there seems to be more general agreement on phagocytosis of the spirochetes immobilized and sensitized by specific antibodies (143, 179). In the classical experiments of Novy and Knapp (151), phagocytosis and intracellular digestion by macrophages of organisms altered by immune serum were very rapid. Kritschewski and co-workers (106) found that phagocytosis is usually an accidental phenomenon in experimental relapsing fever. When organisms were injected intravenously in immune animals only lysis was observed; following injection into skin and peritoneal cavity, some phagocytosis was seen. In tissues, only lysis of organisms and no phagocytosis were the rule. The monocytes seemed less important than the microphages in phagocytosis.

The rôle played by fixed phagocytic cells in relapsing fever is difficult to establish despite the many papers attributing a protective function to the reticulo-endothelial system in this disease (66, 147, 156). Spirochetes have been described within cells by many workers (66, 143, 147, 156), but their work is open to the objection that histological studies are difficult to interpret precisely. Spirochetes are occasionally seen within the Kupffer cells of the liver but none in the macrophages of the spleen (97). Only granular forms are seen in the reticulo-endothelial macrophages of the spleen (97, 147). It is probable that fixed

phagocytes are not very active against living forms of the spirochetes although these organisms are disseminated widely throughout the body (97). Certain macrophages, particularly the splenic cells, may be packed with argentophilic granules; but if phagocytosis of living organisms does occur, spirochetolysis must take place very rapidly because forms recognizable as spirochetes are never abundantly present in these cells (97). The significance of fixed phagocytes in relapsing fever apparently is still an open problem.

One group of workers (97, 147) has presented circumstantial evidence that the spleen is involved in the destruction of the spirochetes. The spleens of severely infected animals did not show enormous numbers of morphologically normal spirochetes while the other organs were loaded with them. In the spleen many intracellular fragments and extracellular granular, degenerated forms were noted. On the other hand, in animals in which not many organisms were seen, the normal organisms were most numerous in the spleen and often could not be seen in other organs. However, these suggestive studies should be confirmed, preferably by other than histological methods, a technic open to considerable danger of misinterpretation.

Kritschewski and his co-workers (107) have made exhaustive studies of the rôle of phagocytic cells and reticulo-endothelial system in resistance to borrelias. It was found that after splenectomy great multiplication of the spirochetes occurs. However, splenectomy is without effect in experimental infections with some species of *Borrelia* (156). There are many observations on the effect of splenectomy or blockade of the reticulo-endothelial system on infection with relapsing fever spirochetes (66, 156), but they do not contribute much of significance to an understanding of the problem. The decreased resistance upon splenectomy or blockade may be a manifestation of decreased antibody formation incident to the removal of large numbers of antibody-forming reticulo-endothelial cells or lymphocytes (53).

*Avian spirochetosis.* Levaditi and Stoel (115) have observed phagocytosis by macrophages of *Borrelia anserinum* in tissue culture. However, Kritschewski and Rubinstein (107) have found phagocytosis to be a secondary phenomenon, occurring after the death or immobilization of the spirochetes. Himmelweit (77) believes spirocheticidins play an active part in the destruction of the organisms in hens. In tissue culture experiments (77) the spirochetes were seen to bore into macrophages from normal spleen cultures but not into fibroblasts and small round cells. In spleen cultures from previously immunized hens the organisms began to bore into macrophages one hour after infection. No phagocytosis was shown by fibroblasts and round cells in these cultures. Twelve hours after infection the spirochetes lay in a braid around macrophages and showed degenerative forms. After a while no live organisms were seen. Upon addition of immune serum to infected macrophages from normal cultures the spirochetes immediately bored into the macrophages. Himmelweit questions whether phagocytosis is the all-important factor, since lytic and immobilizing antibodies are important factors in overcoming the crisis in the infection. Knowles *et al.*

(101), in a recent review of the literature on avian spirochetosis including original experiments on the mechanism of immunity, conclude that immunity in the disease is basically humoral in nature.

*Syphilis and yaws.* A number of investigators have reported finding typical syphilis spirochetes within various cells in tissue sections of natural or congenital human syphilitics (236) or of experimentally infected rabbits (cf. 34). The tissues included liver, lung, and kidney from congenital syphilitics, and testis, liver and kidney epithelial cells, adrenal capsular cells, sweat glands, and cytoplasm of nerve cells from syphilitic rabbits. Bergel (10) reported that phagocytosis of treponemas by lymphocytes and large mononuclears could be demonstrated in the peritoneal cavity of rabbits, guinea pigs and mice inoculated intraperitoneally. However, Chesney (32) states that he has never seen a single instance of phagocytosis of *T. pallidum* either in human syphilis or in experimental syphilis of the rabbit, and he does not attribute an important rôle to phagocytosis in anti-syphilitic resistance. Ferris and Turner (55) have noted no evidence of ingestion of spirochetes by mononuclear or any other cell in cutaneous lesions of syphilis or yaws in man or rabbits. Zinsser and Hopkins (237), in studying the mechanism of natural resistance of mice against *T. pallidum*, observed actively motile unphagocytosed organisms surrounded by masses of leukocytes in the peritoneal cavity as long as 3 days after injection. They could not convince themselves that any significant amount of phagocytosis had occurred. Hoff and Silberstein (81) found some indication that the sera of malaria-treated general syphilitic paralytics contained opsonizing substances for treponema. Beck (7), however, has reinvestigated this matter and concluded that such sera contain no opsonic elements. Occasionally he noted what appeared to be partial ingestion of an organism by a leukocyte but attributed this to an active boring movement of the spirochete into the cell. Most workers, in fact, found the syphilis spirochete in a predominantly extracellular position in the tissues (184). The ability of these organisms to penetrate granulating wounds (35) also speaks against the occurrence of phagocytosis on a very large scale.

Although it is commonly assumed that the fixed tissue elements play an important rôle in defense of the host against syphilis, there is little factual material to indicate that this is true or what this rôle might be. Phagocytosis has not been shown to occur to any great extent in these tissues (184). Although the spleen and lymph nodes usually contain only a few organisms, this may not be due to phagocytosis by macrophages. It may rather be that the extracellular fluids in these organs afford a poor medium for growth of the spirochetes (41, 48, 164). Jungeblut (90) observed that blockade combined with splenectomy in mice in no way altered the course of subsequently induced syphilitic infections. The disease was latent, as in the control animals, with localization of the organisms in the lymph glands.

Phagocytosis may be of great importance in cleaning up the lesions in syphilis and thus assisting in the removal of toxic products from the body. The importance of an increased number of large macrophages in the resolution of syphilitic lesions in the rabbit has been emphasized by a number of investigators (124).

*E. Acquired resistance*

Specific antibodies appear to be primary elements in acquired resistance to leptospirosis and infections produced by species of *Borrelia*. However, until quite recently there was no evidence for the participation of antibodies in the resistance which is acquired in the course of syphilitic infection in man and animals.

*Leptospirosis.* In natural and experimental leptospiral infection the host apparently becomes resistant to infection coincident with the development of specific agglutinating and lytic antibodies during the second week of the disease. The resultant resistance may persist for months or even years paralleling the persistence of the antibodies in the blood. According to Kaneko and Okuda (92) the antibodies are capable of destroying the spirochetes found within the organs of man, with the exception of those in the kidney. Similar results have been obtained in experimental infections in guinea pigs (187) and hamsters (185) whether the antibodies were formed actively or acquired passively. Specific antibodies have a definite therapeutic effect on leptospirosis in susceptible strains of mice if given as late as the fourth day after infection (111).

It is not known definitely whether complement must be present for lysis of *Leptospira* by specific antibodies (185, 219).

Active and passive immunization are quite successful in leptospirosis (219). Immunity may be transferred to the fetus apparently by way of the placenta (219).

It has been claimed that antisera to leptospirosis exert their beneficial effect by virtue of their antitoxic content (219). However, final judgment on this view must await conclusive demonstration of a spirochetal toxin.

*Relapsing fever.* Human cases of relapsing fever characteristically result in long lasting acquired resistance to reinfection (150). Moreover, human immunity seems to be more lasting, relatively, than that observed in the lower forms where after some months it is sometimes possible to demonstrate a certain amount of susceptibility (143). However, second infections may occur in human cases where specific therapy has interrupted the natural processes of immunization (143).

The important rôle of spirocheticidal antibodies in infections with *Borrelia* was first recognized by Gabritschewsky (62) and later put on a firm experimental foundation by the classical experiments of Novy and Knapp (151). It was shown that the spirochetes will remain alive for forty days in blood drawn before the onset of an attack, but in blood drawn during the decline of an attack or after recovery they die out in less than an hour (151). When the organisms were examined *in vivo* during the decline phase of infection, they were observed to lose their motility and become agglutinated, degenerated and granular (151). This also could be observed when blood from an immune animal was inoculated into an infected rat. In rats that had recovered from the infection, Pfeiffer's phenomenon could be produced *in vivo*. Injection of infected blood into such animals was followed by agglutination and granular degeneration of the spirochetes. The course of antibody production and action in human beings seems to resemble

that in experimental animals. It was suggested by Novy and Knapp (151) that the relapse is the consequence of the survival of a few individuals which are resistant to the specific spirocheticidin and which multiply to give rise to a new serum-fast strain. This train of events is repeated in each relapse. After a number of relapses the active immunity developed by the host is sufficient to prevent further invasion of the blood by the spirochetes and an apparent cure results. Antibodies may be transferred passively to the young of man and animals (147).

Some workers believe that immunity in relapsing fever depends on the virulence and numbers of spirochetes introduced rather than on the highly complex antigenic structure of the organisms (97). Many virulent organisms may elicit an active immune reaction from the host while smaller numbers or mildly virulent strains may not arouse the host so markedly (97). However, it seems that the relapse phenomenon is of greater significance for the outcome of this infection, as a sudden shift in antigenicity of the organisms may negate the acquired immunity of the host.

The participation of complement in antibody reactions with borrelias has not been studied. The benefits of active immunization in relapsing fever are less definite than in leptospirosis. The beneficial effects of specific antiserum may be nullified by the failure of the antibodies to correspond to the newly formed antigenic variant.

The rôle of residual infection in immunity to relapsing fever is discussed in Section II G.

*Avian spirochetosis.* The phenomena and mechanisms of host resistance in avian spirochetosis are essentially the same as in relapsing fever.

*Syphilis.* Acquired resistance in syphilis is quite different from that in the other spirochetoses as well as in most other infectious diseases. It perhaps resembles most closely resistance in tuberculosis (168). It is, therefore, pertinent to note the salient features of acquired resistance in natural and experimental syphilis (34, 222).

Compared with most infections, resistance in syphilis is acquired very slowly. Nevertheless, in man reinfection has not commonly been proved to occur (8, 34). In the rabbit it requires about 6 weeks following inoculation for a state of partial resistance to be achieved (51). This resistance at its best is not always complete as the organisms often persist in a latent state in the tissues of rabbits and conceivably of man (34). At some time between the 45th and 90th day following infection the rabbit acquires more effective resistance against syphilitic infection. It appears to be strain-specific in rabbits (38) and possibly in man (5). Resistance is shared by most of the tissues of the body (39) with the eye a prominent exception (39). However, some tissues appear more resistant to the organisms than others (34, 193). Brown and Pearce (24) believe that the testis, bones, skin, and eye successively take up defense of the body and provide by local reaction much of the general physiologic defense. Resistance persists at a high level throughout the secondary or most active period of the disease and into the tertiary stage. During the latter it appear somewhat to wane as reinoculations

are often more successful at this period (5). At its height, the resistance is considered not sufficient to eradicate infection (5, 34, 66, 193), but sufficient to restrain multiplication of small numbers of organisms and prevent the development of an inflammatory lesion. The number of spirochetes in the challenge inoculum is important in determining whether asymptomatic infection or solid resistance to reinfection is manifested in rabbits (129).

Rabbits treated with arsphenamine before the 45th day of the disease can be reinfected successfully as the organisms have been destroyed before producing sufficient active immunity (34). Between the 45th and 90th days, treatment has a variable effect on reinfectability (34). If treatment is postponed until after the 90th day the animal often is refractory to a second infection with the homologous strain of organism providing there is no trauma at the site of injection (34). In monkeys, the earlier treatment is begun the more apt the animal is to yield a positive result upon reinoculation.

The question as to the persistence of active immunity in syphilis following drug treatment is of more than theoretical concern. Chesney (34) believes that animals treated in the late stages of syphilis are biologically cured and at the same time actively immunized. Neisser and Kolle (34, 102, 222), on the other hand, deny that infection has been eradicated from these animals and claim that they harbor latent organisms as indicated by positive lymph node or organ transfer tests. The interested reader is referred to material on both sides of the controversy (34, 102, 193, 208, 222, 236).

What physiological changes in the host are responsible for the increase in resistance in the course of syphilitic infection? Several investigators have noted an enhanced physiological activity of large mononuclear cells during early acute syphilis (124). An inverse relationship between the intensity of the local lesion and the intensity of secondary lesions has been reported and may be related to this enhanced cellular activity (18). However, in the absence of evidence that these cells are engaged in extensive phagocytosis of treponemata, some other function must be assigned them. These cells have been considered to be active in resolving or clearing up the debris in syphilitic lesions rather than in disposal of the spirochetes themselves (124). Other possible activities of the cells have been taken up in previous sections. There remains only the subject of acquired humoral resistance. This subject will be discussed quite thoroughly as it commonly has been discarded as a factor in syphilitic immunity<sup>7</sup>. Although this phase of resistance seems feeble when compared to the powerful influence of acquired antibodies in other infections (89, 143, 168, 208, 236), it is in fact the only concrete factor in resistance to syphilis which has been established up to the present time.

In the past, experiments have been described which suggested that protective antibodies are developed in the course of syphilitic infection. Ebersson (52)

<sup>7</sup> For example, in an editorial in the *Journal of the American Medical Association* for August 23, 1947, it was stated: "Details of the mechanism of the defensive reaction against syphilis are still unknown. *It is definitely not humoral* but is probably a tissue or cellular reaction." (Italics by author).



mixed virulent *T. pallidum* and serum from persons with late syphilis and from rabbits with syphilis of 6 months' duration or longer and incubated them for 2 hours at 36 C. When inoculated intratesticularly into rabbits, these mixtures produced no lesions, whereas control mixtures containing serum from normal rabbits and rabbits infected for less than 6 months produced lesions. Tani and Oginti (202) reported similar results in experiments in which mixtures were injected intracutaneously. Of greater interest, however, are parabiotic experiments performed by Tani and Aikawa (203). They parabiosed rabbits with active syphilis of 9 to 94 days' duration either with rabbits infected with syphilis for 99 to 459 days or with normal rabbits. In general it was found that rabbits joined to immune rabbits showed definite healing of their active lesions while most of those joined to normal rabbits did not show any healing.

Turner (214) has criticized the techniques and lack of suitable controls in previous experiments which led him to his studies on the question. He realized that any test to demonstrate protective antibody in syphilis must be delicately designed so as to bring out the small differences in antibody content that probably exist between the serums of normal and syphilitic animals or human beings. He worked out a technique to satisfy this requirement (214). An emulsion of virulent *T. pallidum* was added to serums from normal rabbits and from untreated immune syphilitic rabbits, infected with an homologous strain of *T. pallidum*, and the mixtures, after incubation at 37 C for 6 hours, were injected intracutaneously into normal rabbits. Typical syphilitic lesions developed at the site of inoculation of the normal serum-spirochete mixtures, while at the sites of inoculation of the immune serum-spirochete mixtures there was either no manifest response or a reaction marked by a longer incubation period and smaller lesions than in rabbits injected with normal serum-spirochete mixtures. Ten out of eleven syphilitic persons with negative reactions to serologic tests for syphilis were positive for protective antibodies by Turner's test (216). Complement seemed necessary to demonstrate the action of these protective antibodies. The fact that positive reactions for protective antibodies were found along with negative reactions to serologic tests for syphilis suggests a probable lack of identity between antibodies for positive serology and those necessary for protection. In any event, these studies indicate that the protective antibodies are associated with a high degree of acquired immunity to the disease.

In recent studies on a larger scale with serums from human beings with syphilis, Turner (212) essentially has substantiated his earlier results: "The serum from syphilitic patients showed a substantial degree of protection as compared with serum both from non-syphilitic hospital patients and from the normal pool. Serum from patients with latent and late syphilis as a group exhibited a higher degree of protection than serum from patients with early syphilis. Serum from patients with secondary syphilis as a group was more protective than serum from patients with primary syphilis, but here the figures are smaller . . ." The results of the protection test did not correlate closely with the complement fixing titer using ordinary beef heart antigen, but no tests were made in which the so-called

Wassermann antibody was removed by adsorption. Turner (212) has no direct information as to whether the antibody would be protective regardless of the route of inoculation of the spirochete-antibody mixture.

In their extensive studies on experimental syphilis in the rabbit, Brown and Pearce (18, 20, 21) observed that the lesions exhibited a relapsing character paralleled by agglutination and degeneration of the spirochetes. Though less regular and marked these observations recall the situation in relapsing fever. Moreover, the agglomeration and lysis of *T. pallidum* are highly suggestive of the operation of specific agglutinating and lytic antibodies such as have been produced in relapsing fever and leptospirosis, and with culture strains of *T. pallidum* (239).

Chesney and co-workers (39) have presented indirect evidence for the humoral nature of host resistance in syphilis. It has been known for some time that the cornea of the syphilitic rabbit does not share to the same extent as other tissues in the general resistant state which develops in that animal during the course of syphilitic infection. It was assumed that this was connected with the absence of a blood supply to the cornea so that the corneal cells either did not receive an antigenic stimulus sufficient to induce immunity or did not receive through the circulation enough of syphilis antibody to endow them with resistance. However, when the cornea was vascularized by previous inoculation with an irritant (39) (dead tubercle bacilli), it manifested greater resistance against a second inoculation with homologous *T. pallidum* than did the normal eye. This result might be due either to exposure of corneal cells in the vascularized eye to a greater amount of antigen with consequent more active cellular response, or to the presence of increased concentration of circulating antibodies. As the rabbits were treated with arsphenamine prior to vascularization, it seems unreasonable to suppose that the eyes had been exposed to an undue amount of circulating antigen. Therefore, the authors were inclined to the view that the greater resistance on the part of the vascularized cornea was more likely due to an exposure to increased circulating syphilitic antibody.

Reynolds (165) cites experiments from the literature which suggested that *T. pallidum* inoculated into immune animals were not only restricted in spread but destroyed locally. He criticized these experiments as dependent on histologic observations for their validity and hence unreliable. In his own studies, Reynolds followed the fate of homologous strains of the syphilis spirochete inoculated subcutaneously into immune rabbits. These organisms did not migrate to the regional lymph nodes but were immobilized and destroyed *in situ* by the immune mechanism of the host, probably by a local antigen-antibody reaction.

Despite the extensive literature recording negative attempts to demonstrate protective antibodies in syphilis (34), the positive and suggestive experiments summarized above force the conclusion that immunity in syphilis rests on at least a partly humoral basis. Possible difficulties in demonstrating protective antibodies are indicated in Turner's work in which these antibodies were shown to be of low titer and probably distinct from the antibodies detected by the usual

serologic tests for syphilis. Moreover, the apparent strain specificity of resistance in experimental syphilis (38) points to the importance of employing homologous organisms for the demonstration of strain-specific antibodies.

Whether the protective antibodies in syphilis exert their effect at least partly by their opsonizing properties is open to further study.

The results of vaccination in syphilis using various kinds of living, attenuated and dead vaccines have been essentially negative (34). There is no evidence for the transfer of immunity from syphilitic man or rabbits to their off-spring (96, 184). However, the positive parabiosis experiments of Tani and Aikawa (203) bring up the possibility of passive transplacental transfer of protective substances in syphilis.

The rôle of infection-immunity or latent infection in acquired resistance to syphilis is discussed in Section II G.

### F. Hypersensitivity

The importance of hypersensitivity in the pathogenesis and immunology of infectious diseases is a question of tremendous theoretical and practical consequence. There are reliable students of disease on all sides of the question, some holding that hypersensitivity is definitely advantageous and even necessary, others that it is decidedly deleterious or at least unnecessary, and still others that it is without appreciable effect for the effective operation of acquired resistance (166, 167, 168, 169, 222). It would be unrewarding to enter into an extended discussion here of this acute controversy. At least some of the difficulty arises from lack of general agreement on the definition of hypersensitivity (167, 222). While it is convenient to explain the pathogenesis and acquired resistance in syphilis in terms of hypersensitiveness, it would be preferable for this concept to rest on a firm factual foundation.

Although skin tests employing antigenic material derived from bacteria, fungi, viruses and helminths have proven useful in the diagnosis of many diseases, this method has not been employed with any great success in any of the spirochetoses. There is no report of hypersensitivity in the literature on relapsing fever. The only report of a skin test in leptospirosis is that of Jacobsthal in 1917 (86). However, his observations were only preliminary in nature and never confirmed. Stavitsky (185) noticed that four or five days after intradermal inoculation of *L. icterohaemorrhagiae* into guinea pigs concentric hemorrhagic lesions often appeared at the site of injection. It is not known whether this represents an allergic response (45) or is due to hemolytic or toxic substances of spirochetal origin concentrated in the injected area by virtue of increased capillary permeability there (137). There are no further data on hypersensitivity in leptospirosis. The luetin test (148) employing killed suspensions of *T. pallidum* as a skin test agent for the diagnosis of syphilis was found to be extremely non-specific. The organic luetin reaction (105) using extracts of mature syphilomas from rabbit testes is apparently more specific under certain conditions.

The exalted or at least altered reactivity of a host upon second contact with a foreign protein of bacterial or non-bacterial origin has been noted repeatedly

(156, 167, 168, 208, 236). Consequently, it is not surprising that the syphilitic rabbit may react differently at a second encounter with *T. pallidum* than at their first meeting. Lisi (119) found that when injections of virulent spirochetes were given to vaccinated rabbits, the lesions were larger and the period of incubation shorter than when injections were given to control animals. Aronson and Meranze (3) studied the lesions produced by injection of tubercle bacilli intracutaneously into syphilitic and non-syphilitic rabbits. The authors concluded that the cellular reaction in the syphilitic rabbits was an "anamnesic reaction", i.e., the cells of the syphilitic rabbits were so modified that injection of an unrelated organism provoked a prompt inflammatory response characteristic of the initial syphilitic reaction. The perivascular focal character of the lesions, the presence of large mononuclear cells and fibroblasts, and the formation of new vessels suggested syphilis; on the other hand, the subsequent appearance of epithelioid cells and of caseation and softening were more reminiscent of tuberculosis.

Rich *et al.* (169) have conducted extensive experiments to clarify the rôle of hypersensitivity in syphilis. Noguchi (148) had shown previously that rabbits injected repeatedly with virulent *T. pallidum* gave allergic skin reactions to a spirochetal suspension ("luetin reaction"). However, Rich *et al.* (169) found immunized rabbits refractory to large test doses of spirochetes without displaying any microscopically discernible inflammatory reaction. In no instance was there the slightest gross or histologic indication of a more prompt or exaggerated hypersensitive inflammation in the immunized rabbits as compared with the controls. On the contrary, after the initial minimal inflammatory response which followed injection of the spirochetes, and which never was more prominent in the immunized than in the control animals, the sites of inoculation in the immunized animals promptly became and remained normal, whereas the lesions in the controls progressed steadily to chancre formation. Rich therefore was led to conclude that although hypersensitivity may appear under certain conditions during certain stages of infection in some individuals, experimentally it had been demonstrated that acquired resistance in syphilis is not dependent upon hypersensitive inflammation; nor does hypersensitivity necessarily develop concurrently with acquired resistance. Acquired resistance and hypersensitivity are distinct and unrelated phenomena.

Gastinel *et al.* (65) recently have presented evidence for the thesis that immunity and hypersensitivity are different manifestations of the same process. He produced both immunity and hypersensitiveness in the same animal by the following procedure: he first reinoculated (without eliciting a local reaction) the testis on the side previously injected; then he reinoculated the other side, promptly producing a chancre with pronounced necrosis, suggesting localized hypersensitiveness of the testicular tissues. Low (123) has considered that "the relative immunity in syphilis is really a hypersensitiveness. . . . Persons with syphilis are protected from reinfection by the local hypersensitive reaction which occurs at the seat of reinoculation." According to this view the organisms are localized at the site of reinfection by the allergic inflammatory response. Rey-

nolds (165) has, in fact, observed such a localization of the organisms of reinfection. However, he attributed this localization to the immobilizing action of specific antibodies on the organisms by a local antigen-antibody reaction, probably agglutination, as has been observed in other infections (166).

The results of the studies of Gastinel *et al.* (65) recall the experiments of Lisi (119) and suggest that hypersensitivity may at times influence the development of pathological alterations in the syphilitic host. The evidence (156, 167, 168) that hypersensitivity may condition the pathological response of the host in tuberculosis and other chronic granulomatous diseases lends weight to the postulated rôle of allergy in the pathogenesis of syphilis. In all of these diseases the rôle of hypersensitiveness is to intensify the response of the host to the pathogen and to act to the apparent detriment of the host. The destructive tertiary lesions in syphilis especially have been considered the result of an altered response (hypersensitivity) toward the spirochetes (193). It is obvious that considerable precise study and agreement on definitions of basic terminology are called for to settle the question of the rôle of hypersensitivity in syphilis.

#### *G. Carrier and relapse states and infection-immunity*

These phases of resistance to spirochetal infection are important but as yet inadequately understood.

*Leptospirosis.* *Leptospira* usually does not give rise to relapses (219). However, there are mildly virulent variants of the organism which do (5). In these so-called "kurzfristigen" leptospiral infections in man the fever curve in general is of the same type as that in relapsing fever.

The symptomless carrier state often is observed in natural and experimental leptospirosis, usually in relatively resistant species of animals such as mice and rats (219). However, individuals of susceptible species may under some circumstances become carriers. According to Tjong (207) the carrier state is established as follows. During the acute period of the infection the spirochetes pass from the interstitial renal tissues through the wall of the tubules into their lumen. The organisms are then swept into the lumen of the distal convoluted tubules and lodge there because of the weak flow of urine and the tortuosities in these tubules. The organisms then maintain themselves by multiplication there and are shed continuously in the urine.

It is well established that the carrier of *leptospira* in the kidneys, whether man, dog, or rat, often has a considerable concentration of anti-leptospiral immune bodies (agglutinins and lysins) in the blood and urine (80, 112, 183). It is difficult to understand how the organisms survive in the urine in the presence of antibodies unless the organisms have undergone antigenic variation and are no longer homologous for the antibodies. However, it has not been shown that *Leptospira* undergoes antigenic variation *in vitro* or *in vivo* to any great extent.

The factors responsible for the latent state in leptospirosis are obscure at present. The relapses due to mildly virulent strains are probably reflections of the mild virulence with its consequent mild stimulus to active resistance on the part of the host, giving rise to inadequate immunity to prevent relapses. The

apparent ineffectiveness of phagocytosis as a defensive weapon in leptospirosis may contribute further to the inability of the body to eradicate the infection.

*Relapsing fever.* Before considering the possible mechanisms of the relapses in this disease, the generally accepted facts of this phenomenon (66, 97, 143, 180) will be stated. For convenience and clarity they are listed:

1. In the course of natural human or experimental infections *Borrelia* produces one or more, usually several, relapses.
2. A typical relapse episode is characterized by the appearance of organisms in the blood, persistence there for many days, disappearance from the blood for a variable period of days and then reappearance in the blood.
3. The number of times this episode is repeated is variable in different species and dependent on many factors.
4. During each episode the antigenic composition of the organism is different from that of strains participating in past or future relapses in that host. Antigenic variants do not recur (181). However, all possible antigenic varieties may not appear in the course of an infection.
5. Upon introduction into a suitable host, a single spirochete may produce all the antigenic variations which characterize that particular strain during infection in that host (181).
6. The succeeding antigenic variants may tend to become weaker in virulence (143).
7. The relapse strains of spirochetes reappear against a definitely rising titer of antibody which is able to confer a considerable amount of immunity against any relapse strain (181).
8. If the antibody-forming mechanism of the host suffers any substantial impairment, as by daily injections of foreign red cells, the relapse phenomenon may be reduced in number of relapses, or entirely absent; and the host may undergo a violent infection (97). If the animal does not succumb to this nearly fatal attack, a single prolonged period of reaction with organisms in the blood ensues, after which the spirochetes cannot be found in the peripheral circulation. Occasionally two severe reactions occur lasting as long in total duration as the single reaction but separated by 1 or 2 negative days. After recovery the resulting immunity is broad in its specificity and enduring in quality.
9. Artificial crises can be produced by injection of specific antiserum, provided it is active against a sufficient majority of the antigenic varieties of the spirochetes involved in the attack (179).
10. The great majority of the surviving organisms are destroyed at the crisis of each attack (97).
11. The antigenic variants may be detected by agglutination or adhesion tests or spirocheticidal action of immune serum (78). Cross-immunity tests may have limited usefulness for this purpose (78).

Some workers have presented evidence which refutes the concept that antibodies are involved in the relapse phenomenon (180). However, taken as a whole, the above data, especially points 8, 9, and 10, support the generally accepted concept that relapses in this disease are somehow bound up with antigen-

antibody reactions. More specifically, relapses appear to be dependent on the inherent ability of borrelias to undergo one or more antigenic phase variations during the course of the disease. The new variant is resistant to the action of the highly strain-specific antibodies elaborated in response to the previous antigenic varieties of *Borrelia*. However, it appears that only a very small percentage of the many organisms participating in an attack are capable of accomplishing this variation as the great majority are destroyed at the crisis of the attack. Presumably the spirochetes which finally succeed in producing antigenic variants are some of those which may leave the blood stream during an attack to seek a haven in the brain, spleen or other tissues from lytic antibodies. Additional antigenic variations account for succeeding relapses until the capacity for variability of the spirochete is spent. Complete recovery then ensues.

It should be stressed that, beyond the stated facts of antigenic variation, the above picture of the pathogenesis of the relapse phenomenon in relapsing fever is at present far too impressionistic. It is difficult to account for all of the facts of the relapse phenomenon solely on the basis of successively distinct antigenic complexes of spirochetes. If the relapses are closely dependent upon antigen-antibody combinations, how does it happen that these reactions become effective at such regularly predictable intervals? Moreover, how can these antisubstances be effective enough to clear the blood stream of enormous numbers of organisms and yet fail to be effective against a certain few? Where or how do the few organisms escape destruction? Also to be explained is the demonstration that succeeding antigenic variants tend to become weaker in virulence and that they appear against a definitely rising titer of antibody capable of transferring immunity against any of the relapse strains. Satisfactory answers to these and other questions require considerable further study. It is a fact, however, that, regardless of the variations in antigenicity or virulence, relapses occur at regular intervals under natural conditions.

Various species of *Borrelia* may become latent in the brain and possibly other tissues of experimental animals during the interval between pyrexial attacks or after the blood is no longer infective (66). Some workers (143) go so far as to state that it is doubtful whether a true cure occurs in relapsing fever, in the sense that infection has been eradicated completely from the body. This is difficult to determine as it seems certain that even though organisms give no indication of their presence, they may remain alive in the tissues for weeks or months (143). The mechanisms responsible for this latency are poorly delineated. Among the possible mechanisms are the antigenic variation of organisms, intracellular habitat of the organisms (coccoid phase?) and consequent protection from noxious influences, change in electrokinetic potential of the organisms which permits them to pass through the blood-brain barrier while antibodies do so only very slowly.

This latent or residual infection is the subject of a heated controversy as to the nature of immunity in relapsing fever (66). As in syphilis, some students connect the immune mechanism with residual infection; in other words, the immune state is maintained only as long as there is residual infection. This argu-

ment will be discussed more fully in the section on infection-immunity in syphilis. However, it may be stated here that supporters of this idea claim that the refractory period of immune animals bears a much closer relationship to the demonstrable presence of viable organisms in the tissues than to the concentration of antibodies in the blood (143).

*Syphilis.* Relapses which occur in human syphilis are often probably related to inadequate chemotherapy with resultant production of drug-resistant variants or to premature treatment before adequate active immunity has been established (34, 193). In extensive experiments on rabbit syphilis, Brown and Pearce (18) found that the lesions exhibited an essentially relapsing character paralleled by cyclic degenerative and agglutinative changes in the spirochetes. In view of these studies, it is possible that the relapsing nature of experimental syphilitic lesions has a basis similar to the relapses in borrelia infections—antigenic lability of the spirochetes and the development of lytic antibodies by the host. However, further study is required to establish this hypothesis.

Symptomless and lesionless infection with *T. pallidum* for many months, particularly in lymph glands (22, 34), but also in one or more internal organs including spleen and brain (34, 184, 209), occurs in experimental infections of the monkey, mouse, and rabbit and probably in human infection. In the mouse, (208) inoculation with syphilitic material is usually without result; no lesions or symptoms develop (208), although the organisms apparently persist and multiply in the tissues (208). This may be tied up with failure of the mouse to develop much hypersensitivity in experimental infections. A similar failure of the rat to develop the characteristic epithelioid cell lesions in tuberculosis has been correlated with failure of this species to develop a high degree of hypersensitivity to the tubercle bacillus (228).

There is a great argument as to whether rabbits or man are ever cured spontaneously or after treatment in the late stages of syphilis, or whether the organisms are always latent in the tissues (34). Some workers (34) have presented evidence for biological cures in rabbits under these circumstances. Cures with arsphenamine and penicillin apparently are possible in the early stages of infection (222). However, it is known that infection may become latent during any of the three stages of human syphilis (193).

The concept of infection-immunity has been developed in connection with a number of diseases: protozoan, bacterial, and spirochetal. It maintains that immunity in these diseases corresponds to a situation within the host in which, together with humoral immunity, there is a latent tissue infection. It is a state of equilibrium between host and invader which, like other equilibria, may be disturbed. The parasite does not make its presence very obtrusive to the host. The result is practically complete absence of tissue reaction. This concept is supported by Neisser and is based on two sets of evidence (34). The first determined that untreated syphilitic apes which were refractory to reinfection could be shown to harbor spirochetes in their internal organs. The second type of evidence supported the view that infected apes treated with various antisymphilitic drugs and thought to have been cured, were at once susceptible to reinfect-



tion, whereas other apes treated but thought not to have been cured remained refractory. Although the first point is well corroborated, Chesney (34) casts doubt on the validity of the second point. The crux of the argument seems to lie in justification of the assumption that animals later shown to be susceptible to reinoculation had been cured of their first infection by treatment, and that those which later proved refractory to re-injection were not cured. Recent studies in rabbit syphilis would seem to controvert Neisser's views and indicate that an immunity not dependent upon persistence of foci of infection may be developed in syphilis. Treatment late in the disease, when rabbits had had opportunity to develop active resistance, abolished disease as shown by negative lymph node transfer experiments, and yet left resistance to reinoculation intact (34). Clinical evidence (34) in man also supports Chesney's ideas. Moreover, Chesney (34) considers Neisser's concept as strange in the light of general immunological principles. One would hardly expect that resistance acquired as a result of syphilitic infection and great enough to protect an animal against large doses or reinfection would vanish at once after the first infection had been eliminated.

There are few data to support further speculations as to the mechanisms of infection-immunity. It is conceivable, for instance, that persistence of organisms in the body might stimulate the maintenance in the peripheral circulation of a titer of antibodies adequate to cope successfully with any spirochetal invader. These organisms might be more or less tolerated without active reaction by the host or might stimulate defensive mechanisms more than is readily apparent. However, in the absence of a solid foundation for the concept it would be wisest to defer final judgment on infection-immunity until supportive data are obtained. For, convenient as it might seem for the depiction of the latent state in syphilis, the concept of infection-immunity suffers from the same serious lack of concrete supportive evidence as it does in relapsing fever.

It is important to know whether patients with latent syphilis are infective, but unfortunately information on the point is meager. Animal experiments (209) indicate that female rabbits with latent syphilis may transmit the disease to healthy bucks.

It is of great practical significance to know whether biological cure of natural syphilis with drugs, that is eradication of infection, results in persistence of active immunity. Opinion on this question is divided (222). It is to be hoped that experiments with penicillin, an extremely effective chemotherapeutic agent in syphilis, will provide an unequivocal answer to this question (222). At the same time, the question as to the necessity for persistence of foci of spirochetes for acquired resistance ("infection-immunity") may be answered.

#### *H: Local and tissue immunity*

It often has been claimed that true tissue immunity apart from circulating antibodies may be developed upon infection with spirochetes, especially the syphilis organism (78, 143, 193, 208, 219, 153). However, there is little sub-

stantial evidence on which to base this claim. The evidence for the existence of local in the absence of general immunity is also weak.

*Leptospirosis.* There are no data to indicate that local immunity is developed in leptospirosis. However, Ono (153) reported experiments which suggested that a true "cellular or tissue immunity" apart from demonstrable circulating antibodies might be operative in this disease. He claimed to have produced a high degree of resistance in guinea pigs by subcutaneous, intramuscular, or intraperitoneal injection or peroral administration of a weakly virulent culture. This immunity was apparent 24 hours after inoculation and lasted for at least 16 weeks. No symptoms or histological changes accompanied the immunization. However, Stavitsky (185) repeated Ono's experiments but was not able to confirm the establishment of the resistant state so soon. Hindle (78) attributes the persistence of spirochetes in the kidneys of rats to "tissue immunity" but presents no further evidence for this concept.

*Relapsing fever.* Data on cellular factors in latency and infection-immunity, in resistance to *Borrelia*, have been presented in Sections II D, and G. However no data have been found to suggest the occurrence of true tissue or local immunity in the course of natural or experimental infections with *Borrelia*.

*Syphilis.* Zinsser (238, 239) has conducted experiments which indicate that local tissue immunity and susceptibility may occur in syphilis. Twenty rabbits were reinoculated into the testes after primary unilateral chancres in these organs had healed. It appeared that the opposite testis could be successfully infected before, during and after the existence of a testicular lesion on one side, but reinfection of the same testis which had apparently returned to the normal state, at periods from 6 weeks to 1 year, was unsuccessful. Kolle (102) confirmed and extended these observations and termed this local resistance "chancre immunity." This type of immunity renders animals resistant only in so far as the formation of cutaneous lesions is concerned; it fails to prevent invasion of *T. pallidum* into the internal organs (34). Therefore, Chesney (34) regards "chancre immunity" as only partial and but a step in the direction of complete immunity. This partial immunity is not sufficient to eradicate the infection but sufficient to suppress the multiplication of small numbers of organisms and thus prevent development of inflammatory lesions (34, 51, 222). Chesney (37) has presented evidence for the wide distribution of the resistant state among different tissues after intracutaneous injection in rabbits. However, local resistance might be associated with local formation of antibodies (64) or their concentration in the local inflamed area (137). On the other hand, more vigorous mobilization of physiologically hyperactive cells in a tissue upon second exposure to an organism (127), may underlie the enhanced local resistance to infection. Manifestly, the entire problem of local as distinct from general resistance requires further careful study.

Until recently, in the absence of evidence for the rôle of humoral antibody in syphilis immunity, it usually has been stated that immunity rests in the cells or tissues. However, with the acquisition of evidence of several types for the

development of protective antibody in syphilis (39, 52, 212, 214, 215), it is only reasonable to demand that comparable evidence be presented for a concept of immunity of cells as distinct from humoral antibodies.

### *I. Diet, fatigue, trauma, temperature, and light in resistance*

The manner in which these factors may influence host resistance is difficult to ascertain as they may be acting upon the microbes, the host, or both.

*Leptospirosis.* There was no discernible effect on leptospirosis in guinea pigs of raising their rectal temperature to 40–42 C by incubation in a hot chamber at 42 C (160).

*Relapsing fever.* No data were noted on these factors in relapsing fever.

*Syphilis.* The data on dietetic factors in syphilis are meager. It has been claimed that green fodder protects rabbits to some extent (193), but the factor in the fodder is unknown.

It has been claimed that physical strain may predispose man to cardiovascular lesions, arthritis, aortitis, and other complications of syphilis (193).

Trauma predisposes to syphilitic infection in rabbits in an unknown way (35, 36). It is known that the organisms settle out and multiply profusely in inflammatory areas (36). Whether this is a consequence of increased capillary permeability in the local region (137) or some other mechanism is unknown.

Temperature may influence strikingly the course of syphilitic infection. It has been noted that the period of incubation of experimental rabbit syphilis is increased during the summer (193). Artificial short wave fever may destroy the organisms and cause healing of the lesions in experimental or natural syphilis (28). The mechanism of the beneficial effect of short wave fever in rabbit syphilis (28) is not completely known. It may activate the body defenses through the effect of heat on the adrenal cortical-pituitary system in reaction to stress (229). Bessemans (11) claims that *T. pallidum* in experimental infection displays a predilection for localization and multiplication in those tissues having the lower temperatures in the body.

In general, the reaction to syphilitic infection is increased in proportion to the amount of light and the constancy of exposure of rabbits to that light (23). The mechanism is unknown, but it was observed that the light modifies the physical constitution of normal rabbits, and the organs most affected were those concerned with the response to syphilis. Brown and Pearce (23) believe that the effects are probably manifestation of functional activity closely related to changes in physical constitution.

Ultra-violet radiation lowered the weights of normal and syphilitic rabbits (72), but the significance of these observations for an understanding of syphilitic resistance is questionable.

### III. DISCUSSION

The increasing number of factors which must be considered in interpreting the data presented caution us that we are first beginning to acquire enough information to start to put together the picture of the pathogenesis of and host re-

actions to infections. Nevertheless, some generalizations from these data would seem to be justified if only for their heuristic value.

It is clear from this survey of the main features of the spirochetoses that in general they conform very closely to the well-established principles of pathogenesis and host resistance in infectious diseases (6, 66, 156, 166, 168, 197, 208, 236). What, then, is so unique about spirochetes and the diseases they produce? Is it true, as Henrici (74) has noted, that "infectious diseases present specific earmarks which are determined by the species which cause them; nosology reflects taxonomy"?

There seem to be three groups of pathogenic spirochetes from the standpoint of nosology as well as taxonomy (66). The treponemas, including the organisms of syphilis, yaws, rabbit venereal spirochetosis (135, 218), and possibly pinta (82), are well-adapted tissue parasites showing highly individual species adaptation to man and rabbit (in case of venereal spirochetosis) and giving rise to incompletely developed acquired resistance to reinfection. The borrelias are blood parasites which produce the relapsing phenomenon most strikingly. They are mainly parasites of lower animals which probably infect man accidentally. Antibodies against them resemble bacterial antibodies in their great specificity and concentration. The leptospiras infect the tissues and blood and are widely promiscuous in species adaptation (139), infecting a variety of lower animals and man. They produce acute diseases which usually terminate quickly in death or solid and enduring resistance to reinfection. Of the spirochetes, the leptospiras most closely resemble bacteria in general biological properties.

There seems to be an essential similarity in the pathogenesis and host resistance in spirochetal infections. This similarity involves the cyclical character of symptomatology and lesions in these diseases and the parallel cyclic agglutination and degeneration of the spirochetes, a phenomenon most regularly and readily observed in relapsing fever. In acute leptospirosis there is only one attack recovery from which is preceded by agglutination and lysis of the organisms. However, in a type of human leptospirosis caused by mildly virulent variants, clinical relapses commonly are observed (5). In experimental syphilis of the rabbit, the concurrence of retrogression of the lesions with degenerative changes in the organisms have been described by Brown and Pearce (18-21), and compared to similar phenomena in relapsing fever. Human syphilis is particularly characterized by a series of progressive and retrogressive phases.

Kligler *et al.* (100) have suggested that the characteristic antigenic and pathogenic properties of trypanosomes were conditioned by the predominantly lipoidal type of cell low in glucoside, as compared to the usual bacterial cell which is mainly glucosidic and low in lipid. They cited the tubercle bacillus with its high lipoidal content (2) as another example of a microbic cell against which immunizing antibodies are produced slowly and with difficulty. The similarity in the pathogenesis of trypanosomiasis and spirochetoses might be due at least partly to a common lipoidal Wassermann antigen which has been demonstrated in trypanosomes and spirochetes (226). It may be surmised further that the "antigenic inertia" of *T. pallidum* and possibly other spirochetes is a reflection

of their high lipid content. This assumed low antigenicity of spirochetal lipids might be due to their low solubility in body or intracellular fluids and similarity to antigens present in normal tissues. This would be in accord with the great difficulty of obtaining auto-antibodies (120). The failure of lipids to elicit an active inflammatory response also might contribute to their poor antigenicity. The protective antigens may be subsurface and dependent on breakdown of the spirochetal cell to make them available to antibody-forming cells. In line with this idea is the observation that spirochetes have lipid surfaces (144). Slow multiplication of the organisms with low production and slow release of protective antigen may also underlie the meager and slow protective antibody formation in syphilis. The possible relationship of lipoidal antigens of the Wassermann type of microbic origin to the antigenic and pathogenic properties of the organisms seems worthy of careful study.

Many authorities (32, 39, 97, 101, 107, 212, 214) agree that on the basis of present knowledge immunity in spirochetal diseases seems basically humoral in nature. Chesney (32) states: "I doubt that phagocytosis is the primary element in host resistance to syphilitic infections. I am much more inclined to think that such resistance is dependent upon humoral factors although for a time the evidence of humoral antibodies was nil." Admittedly, the resistance attributable to circulating antibodies in syphilis seems weak by comparison with that in other diseases. Nevertheless, at this time it is the only mechanism of acquired resistance which has any foundation in experimental studies of syphilis. Kritschewski *et al.* (106, 107), on the basis of some of the most recent experimental evidence, conclude that immunity in relapsing fever, avian spirochetosis, and syphilis is primarily due to destruction of spirochetes by specific lytic antibodies with phagocytosis of the organisms being only an accidental or secondary phenomenon. The importance of specific antibodies in acquired resistance to leptospirosis is clear.

There does not seem to be the obvious joining or cooperating of cells in immune activities in spirochetal infections that there is in many bacterial infections (89, 208, 236). However, in view of the new trends in the study of the contribution of the host cell to resistance to infection<sup>8</sup>, a full understanding of the rôle of the cell in spirochetal infections must await further study.

There is no major conceptual difficulty in transposing the picture of spirochetal resistance into the framework of antimicrobial resistance. The basic factors, antibodies and cells, are the same although employed to different degree and in different ways than in other infections. There is nothing particularly unique about the importance of antibodies in spirochetal resistance. Rich (168) has remarked: "In the attainment of her ends Nature may be prodigal of materials, but she is rigorously economical of methods, and in one infection after another, whether caused by bacteria, fungi, filtrable viruses or rickettsiae acquired resistance has been found to depend upon antibodies." The uniqueness would appear to lie rather in the ability of lytic antibodies to destroy the spirochetes with little or no apparent aid from phagocytic cells. The resemblance

<sup>8</sup> See references 41, 68, 88, 91, 127, 134, 164, 166, 173, 191, 200, 227, 229.

between extracellular lysis by antibodies of spirochetes, generally considered gram-negative, and similar lysis of gram negative typhoid and cholera organisms is particularly arresting. One is tempted to suspect a common chemical factor for this similarity. This idea may be related to the recent demonstration of a difference in the surfaces of gram negative and gram positive bacterial cells; the former are devoid of a protein-ribonucleate complex which the latter cells possess on their surfaces (48).

Goodpasture (68) in a discussion of the cell-parasite relationship in bacterial and virus infections has noted that "those infectious agents that are able to

TABLE 1

*Outline of some problems in the study of the mechanisms of host resistance in spirochetal infections*

- 
- I. Parasite factors*
- A. Morphology and life cycle.* Significance of coccoid bodies.
  - B. Electrokinetic potential.* Does it change in sign in spirochetes? If so, how? Importance of potential in relation to blood-brain barrier and phagocytosis.
  - C. Methods of accurately counting spirochetes.*
  - D. Cultivation of Borrelia and T. pallidum.* Growth factors and metabolism, especially as related to non-specific inflammation in support of growth of *T. pallidum*.
  - E. Antigenicity and chemical composition.* Relation to pathogenesis of infection.
  - F. Toxic and aggressive substances.* Presence of hyaluronidase, fibrinolysin, leukocidin, coagulase.
  - G. Tissue tropisms.* Do Borrelia and Treponema exhibit a tropism for brain tissue?
  - H. Variation.* Is it induced or spontaneous? Does it occur in Leptospira and Treponema, and is it related to relapses and carrier state in these infections?
- II. Host factors*
- A. Natural resistance.* What are the mechanisms of age and species resistance? Is antibody formation a crucial factor in natural resistance to leptospirosis?
  - B. Fixed cells.* How often does phagocytosis occur spontaneously without opsonins?
  - C. Antibodies.* Is complement necessary for lysis of spirochetes?
  - D. Hypersensitivity.* Is it necessary or helpful for operation of acquired resistance in syphilis?
  - E. Mechanism of carrier and relapse states.*
  - F. Infection-immunity.* Is it necessary for acquired resistance and, if so, how does it operate?
- 

survive and persist even to grow, within living cells are most apt to escape their antagonistic effect (*circulating antibodies*) (italics by author); and taking advantage later of a lowering of the antibody concentration and other favorable factors, then may renew the attack . . . and indeed infections with these agents . . . may be of relatively long durations, and in some cases recurrent." Thus far spirochetes, at least in their characteristic spiral morphology, have seldom been noted intracellularly. Indeed, the organisms are seen mainly extracellularly *in vivo* and would seem at most to be only facultative intracellular parasites.

The importance of the antigenic stability of the microbe to the effective operation of acquired resistance should be stressed. Antigenic variability is a property of the parasite whereby, by a change in antigenicity, it may frustrate completely

the solidly acquired resistance of the host. This antigenic lability may render it difficult or impossible to actively immunize a host effectively. It may account also for the failure of chemotherapy. After a non-sterilizing dose of drug, the acquired resistance of the host cannot complete the task of destruction. It is suggested by studies of *Borrelia* as well as trypanosomes that variability of organisms may include the ability to change the sign of its electrokinetic potential.

As has been noted by others (89), many characteristics of trypanosomiasis (4, 198, 199) are very much reminiscent of those of spirochetoses. The relapsing character of trypanosomiasis, particularly its connection with the development of antigenic variants and specific lytic antibodies (197, 201) comes to mind first, as it has been considered here as a typical feature of spirochetoses. The morphology, serological cross-reactivity of spirochetes and trypanosomes (46), presence of a common Wassermann antigen (108, 226), positive zeta potential of some strains of spirochetes and trypanosomes (15, 16, 42), are further indications of a possible biological relationship between these two groups of microorganisms. Whether this relationship has evolutionary significance remains to be determined by further investigation.

In the field of spirochetes and spirochetoses many problems await study. Some of them are summarized in table 1. Interestingly enough, many of them, including infection-immunity, carrier and relapse state, relation of hypersensitivity to problems of infection and immunity, are being debated also in relation to other microbial agents.

In view of the singular properties already recognized in the biology and chemistry of the pathogenic spirochetes, the author commends these organisms as an intriguing virgin and fertile field of study for general microbiologists as well as for immunologists.

#### IV. CONCLUSIONS

1. The pathogenic spirochetes and spirochetoses conform very closely to the established principles of pathogenesis and host resistance in infectious diseases.

2. The spirochetes are heterogeneous and from the standpoint of pathogenesis of the infections they cause, as well as taxonomy, may be divided into treponemas, borrelias and leptospiras.

3. There seems to be an essential similarity in the pathogenesis and host resistance in spirochetal infections which involves the cyclical character of symptomatology and lesions in these diseases and the parallel cyclic morphologic changes in the spirochetes.

4. On the basis of present knowledge antibody formation with lytic properties seems to be the most important factor in acquired resistance in spirochetoses. Phagocytosis apparently is a secondary or accidental phenomenon.

5. Some of the characteristics of spirochetes such as antigenic variability, and capacity to produce relapses may be related to their possession of the ubiquitous Wassermann type of lipid antigen.

6. The resemblance of trypanosomes and trypanosomiasis to spirochetes and

spirochetoses on the basis of points 3, 4, and 5 suggests that these two groups of microorganisms may be biologically related.

7. The spirochetes must be commended as a virgin field of study for general microbiologists as well as immunologists.

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# THE MOST ABUNDANT GROUPS OF BACTERIA IN SOIL<sup>1</sup>

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The microflora of soil has been under investigation for at least 70 years; and yet there is at the present time no publication which sums up the existing information in a form sufficiently concise to serve as a guide to any one who is beginning research in soil bacteriology or who has not had the opportunity to follow the literature on the subject in its year-by-year development. A beginner in this field is apt to be assigned the task of plating one or two samples of soil; but after the colonies have developed about all he can do with them is to count the numbers—a matter of little significance in soil bacteriology. He may speculate as to what the various organisms are, and which kinds are of importance; but unless he is associated with someone well versed in the field, he has difficulty getting the information in a fairly concise form.

## *The Significance of Soil Flora Studies*

Before giving much time to studying this subject, one is naturally interested in the question whether such a study may have any conceivable present or future bearing on soil problems of a practical nature. It must be admitted right at the start that it has no such bearing at the present time. Most of the soil bacteriological methods that have been pursued in the past have been of an entirely different nature; in fact Lochhead with his associates (29, 30) have sometimes seemed to offer about the only assistance to the present author (6, 9, 10, 15) in his advocacy of the soil flora method of approach. Nevertheless, it still seems that, considering how much remains to be learned about the general soil flora, and how few practical lessons to agriculture have been obtained from the other, more intensively pursued, methods of investigation, practical results may some day derive from studies of the sort outlined here. Before taking up the main subject matter of the present paper, however, it seems well to discuss other types of soil bacteriological investigation that have been followed in the past.

*Investigations of the nitrogen cycle.* The importance of nitrogen transformations and the probable agency of bacteria therein was appreciated in early days of bacteriology; and investigators such as Winogradsky (46), Omelianski (32) and Beijerinck (1) gave much attention to the special groups—nitrifiers, denitrifiers, nitrogen-fixing organisms, and ammonifiers—which take part in such processes. These classic investigations were fundamental, and the information they furnished as to the importance of bacteria in soil is today regarded as elementary. They led to the isolation of the legume nodule bacteria and to the use of these organisms for soil inoculation—which is sometimes regarded as the one significant change in soil practice which has resulted from bacteriology.

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They also explained the reasons for many agricultural practices, such as crop rotation, composting, and the like, which were already in use before bacteria were known; and they have taught us improved methods for composting or green manuring so as to avoid harmful effects from crop residues in soil.

Incidentally, these early investigations revealed so many kinds of organisms unable to grow on ordinary media that the plate count was quite discredited because it probably represented only a small part of the actual soil flora; and there seemed no valid reason for intensive studies of the bacteria that were able to develop on such plates.

*The Remy-Löhnis method.* A natural outgrowth of this last mentioned consideration was the suggestion of methods depending on other principles than plating. One of the first and most important of these was proposed by Remy (35) in 1902. He made an attempt to determine the physiological functions of a soil by placing weighed amounts (usually 10%) in sterile solutions of known constitution into 1% peptone solution in order to measure ammonification by determining the ammonia produced in a given length of time; and similarly into other solutions favoring respectively the nitrifying, denitrifying and nitrogen-fixing bacteria. Almost simultaneously, Hiltner and Störmer in 1903 (20) proposed a similar method, but inoculated with successive dilutions of soil down to 0.001 mg., and determined the amount of chemical change produced by the greatest dilution which would allow the reaction to occur. These two methods were compared and contrasted in the literature, each vigorously defended by its exponents; but the Hiltner and Störmer method was taken up by few other than its authors, while the Remy method, largely because of its advocacy by Löhnis (31), came into quite wide use. Löhnis modified the method slightly, chiefly by using soil extract as the basis of the solution in which the physiological tests were made. After years of work, principally in Germany and America, it became evident that the results obtained did not parallel actual conditions in the field, and the method gradually fell into disuse. It was illogical, of course, because the biological activities taking place in one of these artificial media were not necessarily the same as those occurring under natural conditions.

About the same time that Remy proposed his method, Withers and Fraps (18, 51) in the United States, developed a similar method. It differed from the Remy procedure in that the ingredients whose decomposition was to be studied were added to a standard soil which was then sterilized and inoculated with the soils under investigation. Logically this was an improvement over the Remy method, as sterile soil presents more natural conditions for the growth of soil bacteria than do solutions. This method was further developed by Stevens and Withers (40) but never came into wide employment. It still presented the theoretical objection that study was being made of a mixed flora, of which entirely different species might predominate under natural conditions; and in actual practice it gave no better correlation with field conditions than did the unmodified Remy method. It was soon dropped, the more willingly, because it presented greater technical difficulties of analysis than did the solution method.

*Microscopic methods.* In 1917 the writer (7) proposed a method of examining

soil (stained with rose bengal) under the microscope to show bacteria. For certain purposes the method is interesting, but it was never claimed to yield practical results, nor to be of much significance in itself. It is not even a satisfactory means of counting bacteria in soil, as it requires more numerous bacteria than those in ordinary field soil in order to yield a fairly reliable count. It has, however, contributed one point of value: it has shown that plate counts, made under proper conditions, although smaller than total counts of bacteria in soil, are not so far from correct as some soil bacteriologists had predicted.

The method, with slight modification, was taken up by Winogradsky (48, 49, 50), when he resumed soil investigations in the 1920's, and made part of his "direct" method of studying soil bacteria. He drew important conclusions from it, to be discussed in the following pages; but most of his work was with other methods. The whole of his procedure does not seem to have been taken up by anyone else—perhaps because it was complicated, or possibly because his description of the steps was too vague for easy following by others.

A more important modification of the microscopic method was made by Rossi (37) and later strongly advocated by Cholodny (2, 3). This method is to bury a slide (wholly or partially) in soil and to stain the film of microorganisms which becomes attached to its surface after a short incubation; but because its results are hard to put on a quantitative basis, it does not seem likely to be of practical value.

*Soil deficiency tests.* One of the steps in Winogradsky's "direct" method (50) was to mix soil with mannitol and water into a sort of paste, and to mold it into a plaque, upon which colonies of *Azotobacter* develop spontaneously on incubation. Sometimes these colonies fail to appear, and this was found in many cases to be due to phosphorus deficiency in the soil. Nearly 15 years previously Christensen and Larsen (4) had proposed the growth of *Azotobacter* as an indicator of deficiency of this element; and Winogradsky seemed to have a simpler method for thus using it. For several years, therefore, the method was extensively investigated, in comparison with chemical tests of soil, plant tests, and growth of other microorganisms (usually fungi) as indicators. It seemed for a time that one or more of these methods might prove useful; but the final outcome of the work has been rather disappointing. None of the methods for employing microorganisms as indicators of soil deficiency has proved promising enough to come into general use.

*Present tendencies.* But for the stimulus which has been given soil bacteriology by the study of antibiotics (see next paragraph), one might almost characterize its present tendency as being one of defeatism. All the above methods have been tried in the hope of getting practical information for agriculture, but except in one or two instances they have been found wanting. What more is there to do? To judge by the recent decrease in number of papers in soil bacteriology (excluding the subject of antibiotics) the general answer seems to have been: Nothing. Rather than take such a discouraged point of view, however, the present writer prefers to think that we may well get worthwhile results by abandoning short-cut methods, and going back to a laborious pure culture study of the general soil

flora, in the hope of learning the functions of the bacteria, one by one. Certain encouragement for such a procedure is derived by considering recent discoveries in the field of antibiotics.

*Studies of antibiosis.* Of recent years an entirely new aspect of soil bacteriology has developed which is certainly practical—although not agricultural. The studies of Fleming (17), Dubos (16), Waksman (44), and others have introduced the field of antibiotics. This subject is certainly not soil bacteriology in its strict sense; and yet it is such an important one that it is engaging the attention of more soil bacteriologists than any other field. It is not part of what are here termed soil flora studies; and yet the very fact that organisms of such unexpected practical value as those producing antibiotics have been picked up from soil plates suggests that there may be other organisms able to grow on these plates, which are still unstudied, but which may be of great importance in other unforeseen ways. In other words, the study of antibiosis indicates, as nothing else has yet done, the importance of learning more about the general soil flora. Little enough is yet known along that line, and the present review is presented in the hope of stimulating interest in this neglected field.

#### *Methods of Classification*

In beginning a study of the soil flora, the first consideration must be the classification of soil microorganisms into broad general groups. Three methods of general grouping appear to the author to be worth considering here: classification by botanical groups; classification by adaptation to laboratory media; Winoogradsky's grouping into zymogenous and autochthonous types. Each of these methods will be discussed in turn.

*Classification by botanical groups.* This method of grouping seems the most natural to the biologist; yet from the standpoint of soil bacteriology it is not necessarily the most satisfactory. Such a classification is essentially as follows:

- Higher fungi
- Actinomycetes
- Eubacteriales:
  - Non-spore-formers
  - Spore formers.

In practice such a classification presents certain difficulties. Aside from the fact that no attention is given to function of the organisms, it also has to be recognized that some of the divisions are not clear-cut. The Higher Fungi (molds) and the spore-forming bacteria (*Bacilliaceae*) are easily recognized, but there is no sharp distinction between the non-spore-formers and the Actinomycetes. The typical members of the latter group (*Streptomyces spp.*) are easily enough recognized; but the gradation, through intermediate forms with meager branching if any, into the typical non-spore-former, is so gradual that no sharp line can be definitely recognized.

*Classification by adaptation to laboratory media.* For practical purposes, the soil bacteriologist often thinks of soil organisms as falling into two groups: (a)

those growing on ordinary media (e.g., gelatin, or peptone media); (b) those requiring special media, or failing to grow at all under laboratory conditions. Such a classification does not go very far, but it does have certain very distinct advantages. It puts the autotrophic bacteria (like the nitrifiers) into one class, and the general decomposition bacteria (ammonifiers, etc.) into the other; and considering the difference in the methods for studying the two groups, this distinction is of practical value in the laboratory. On the other hand, the distinction is no more clear-cut than that between the Actinomycetes and the non-spore-formers. The term "ordinary media" is far from definite; and it is possible to make various modifications of ordinary peptone formulae which can adapt them to organisms formerly unable to grow under such conditions. Furthermore such a classification leaves one in doubt what to do with bacteria that grow *best* on some special synthetic medium but do grow after a fashion on ordinary peptone agar.

*Winogradsky's grouping.* Probably one of the most significant ideas that has ever been introduced into soil bacteriology is Winogradsky's (50) grouping of soil microorganisms into "zymogenous" and "autochthonous" types. It is interesting that this Russian bacteriologist, who in his younger days (46) first showed how to grow autotrophic bacteria in the laboratory, in his later years has contributed such an important conception as the distinction here discussed. The distinction in question is best understood by substituting for "autochthonous" the more familiar term "indigenous" or even the still more English word "native". This group of indigenous bacteria may be regarded as always numerous in soil and not fluctuating much in numbers, carrying on activities which require no nutrients or sources of energy other than those normally present in soil. Winogradsky regarded them as primarily small cocci—a point to which the present author (as discussed below) takes exception without, however, questioning the essential validity of Winogradsky's conclusion. The "zymogenous" flora, on the other hand consists of the actively fermenting forms which require for their activity ingredients that are quickly exhausted; hence these organisms may, under proper conditions, increase rapidly to large numbers, and then equally quickly return again to such low numbers as not to be detectable by ordinary analytical methods.

This broad grouping of soil bacteria seems fundamental. It bears no direct relation to ability to grow on laboratory media, and none whatsoever to botanical groups; but that does not detract from its value. Indeed, the very fact that it cuts across the botanical groups makes it possible to use them for further subdivisions, which prove quite useful once the fundamental separation of zymogenous and indigenous types is recognized. Such a classification will be followed here.

### *The Zymogenous Flora*

The zymogenous types, as above mentioned, are those which take part in the rapid fermentative processes, therefore increasing to large numbers whenever furnished with the special nutrients to which they are adapted, and then, after



the process is complete, subsiding to minimal numbers until another occasion for active growth occurs. It can readily be understood that this group includes those bacteria which take part in the transformations of nitrogen as well as most of the other processes by which organic matter is made available to plants. The organic material in question is not normally present in soil, and when added to soil rapidly disappears; it undergoes successive stages of decomposition, and as each stage calls for its own type of microorganisms, it can be seen that its incorporation in soil may stimulate successively various groups of zymogenous species. For practical purposes we can divide this flora into those organisms which require special media for laboratory cultivation and those which grow on ordinary media.

*Organisms requiring special media.* Prominent in this group are to be mentioned: the nitrifiers, the nitrogen-fixing bacteria, and cellulose decomposing organisms. All three groups, although among the most important microorganisms of soil, occur naturally in such small numbers that they can be obtained in plate culture only after repeated transfers through enrichment media. Methods in use for obtaining them are still essentially those worked out in the early days of soil bacteriology by Winogradsky (46, 47) and Omelianski (30). The nitrifiers are distinctly autotrophic, and do not ordinarily grow on organic media; in fact, it was formerly supposed that they would not live in the presence of organic matter. The nitrogen-fixing and cellulose-decomposing bacteria are not autotrophic, as they require an organic source of energy, and they are not quite so poorly adapted to ordinary media as are the nitrifiers; nevertheless it is ordinarily necessary to use special media for their enrichment and isolation.

Among other bacteria that fall in this group should be mentioned the sulfur-oxidizing organisms, and the acid-fast forms. The latter are specially interesting, as some insist (e.g., H. L. Jensen, 24) that they belong in the genus *Mycobacterium* with the tubercle organism. Their significance in soil seems questionable, since the best known forms seem to be primarily concerned in the decomposition of hydrocarbons (e.g., paraffin), a process which probably is not important in ordinary agricultural soils.

Because of the small numbers in which these organisms requiring special media occur in normal soils, little will be said about them here. The importance of some of their activities is manifest; but their study is undertaken mostly by specialists; and the general student rarely has occasion to try to isolate or to identify them. When such methods are wanted one should consult references cited in text books, as that of Waksman (43), for instance.

*Organisms growing on peptone or gelatin media.* It can be said that the zymogenous types which grow on ordinary bacteriological media are ammonifying forms, or at least organisms that take part in the various stages of degradation of organic matter, even though not all of them result in its final conversion into ammonia. These organisms may be classified according to botanical groups as follows.

(a). *Higher fungi.* The majority of the higher fungi found in soil are commonly referred to by the indefinite, but sometimes convenient, term "molds". They

belong to several families of fungi, and a great variety of species have been identified. Publications describing such forms have appeared in the past; for example, C. N. Jensen (23), Waksman (42, 45) and others (19, 22, 28, 33, 34, 41). These forms do not ordinarily appear on the same plates as those designed to develop the bacterial flora, because of the predilection of molds for rather acid media of high carbohydrate content. It is, however, very easy to prepare media which bring forth numerous colonies of such fungi from almost any soil; the chief difficulty lies in interpreting plate counts in terms of actual mold activity in soil. It is generally assumed that the great majority of the mold colonies come from spores; and it is obvious therefore that a single profusely sporulating fungus, even after passing into an inactive state, might give a higher count than numerous individuals of an actively growing, but non-sporulating fungus. It is hard therefore to get a good idea of the extent of vegetative mycelia in the soil, especially considering that the microscope is of little help. The writer's most commonly employed microscopic method (11) for demonstrating bacteria in soil fails to show molds; and although modifications of the microscopic method (7, 8, 27, 37) have been devised which do show fungi, no one has devised a method for putting such results on a quantitative basis.

Although we lack the data, therefore, for definite quantitative statements, the rôle of fungi in soil seems to be fairly evident. Under ordinary conditions they probably exist primarily in the form of spores; but when supplied with organic matter (especially vegetable) under acid conditions, or conditions where high H-ion concentration can occur during fermentation, the fungi become active. Whether certain species take part in certain stages of this fermentation and others in other stages has not yet been definitely established. Presumably that is the case. In any event, the fungi definitely belong to the zymogenous flora of soil.

(b). *Actinomycetes*. Much of what has just been said about the true fungi, can be said for the intermediate group, actinomycetes. The true actinomycetes are filamentous in vegetative form, yet colonies on the plates arise in most cases from spores, not from filaments. The filaments are almost as difficult to demonstrate microscopically as in the case of higher fungi, although the Rossi-Cholodny (2, 3, 36) method does show them in a semi-quantitative manner. Actinomycetes are presumed to take part in much the same kind of activity as higher fungi, but prefer neutral or weakly basic conditions rather than acid.

The numbers of Actinomyces colonies that develop on plates from soil is surprisingly constant. The writer has plated countless soil samples during the past 40 years, and has rarely found an Actinomyces count (i.e., presumably a spore count) of less than 5 million or more than 30 million per gram. It seems strange, indeed, that a spore count of a zymogenous group of organisms should be so large and so constant; and it is easy to think of the actinomycetes as being, rather, a group of indigenous species. Nevertheless the writer's considered opinion is that such forms, although indigenous to soil in the ordinary sense of the term, are not "autochthonous" in the sense meant by Winogradsky. They are organisms whose vegetative activity seems to occur only when favorable conditions allow

spores to germinate and to produce mycelia. Perhaps we should call such sporogenous organisms (molds, actinomycetes, as well as spore-forming bacteria) "semi-zymogenous".

It is interesting in this connection to remark that Winogradsky was undoubtedly looking, in part, at Actinomyces spores in his soil preparations when he thought he was studying the indigenous flora. He remarks (50) that the bacteria of the zymogenous flora are primarily large rods, those of the "autochthonous" flora cocci. Now the writer has examined soils from all over the United States, and has never found true micrococci in any abundance; it seems hard to believe that the soils of France can be as different as Winogradsky's statement seems to indicate. The only coccoid organisms observed by the writer have been the very abundant Actinomyces spores and the less common coccoid forms (arthrospores?) of the group recently designated (15) *Arthrobacter* (called *Corynebacterium* by Jensen, 24). Accordingly it is not impossible that a large part of the "autochthonous" forms seen by Winogradsky under the microscope were actually Actinomyces spores.

Species identification among this group has proved difficult. More will be said about this in the later section of this paper dealing with methods of identification of common soil bacteria.

(c). *Spore-forming bacteria*. Most prominent among the spore-forming bacteria in soil are the strongly proteolytic species, *Bacillus cereus*, *B. mycoides*, *B. megatherium*, and one or two others. These forms are the most common rapid gelatin-liquefiers of soil origin and have been found by the writer (5, 6) to comprise about 10% of the colonies that develop on gelatin plates inoculated with soil. Other non-proteolytic or less strongly proteolytic spore-formers exist in soil, but are infrequently found on ordinary plates. To demonstrate their presence it is necessary to heat the soil (or soil infusion), before plating, to a temperature sufficient to kill vegetative rods. These last mentioned types are therefore rarely encountered by the student of general bacteriology.

Even before Winogradsky proposed the term "zymogenous" and pointed out the significance of that part of the soil flora, it was realized what must be the function of these spore-formers. It was pointed out (5) that the great constancy of bacterial spores, with but rare occurrence of vegetative forms, must mean that these very active proteolytic species remain normally in soil in inactive form, germinating and multiplying for brief periods only when supplied with proper nutrients. In fact, it was apparently the present writer's conception of the activity of spore-formers which suggested to Winogradsky that other organisms might act similarly and hence comprise a zymogenous flora. The conception is now generally accepted as correct.

Spore-formers have been studied from the taxonomic angle more thoroughly than any other soil forms. There is still some dispute as to where to draw the lines between species in this group; nevertheless, it is true that species identification is easier among them than in the case of any other group of soil bacteria. Methods for the identification of the most common species are given below (p. 267).

(d). *Non-spore-forming bacteria*. Apparently the bulk of soil bacteria (at least so far as concerns the flora developing on ordinary plates) do not belong among the zymogenous types. Almost the only non-spore-formers of the zymogenous type are the fluorescent pseudomonads. They may constitute a single species (*Pseudomonas fluorescens*) or a group of closely related species. The writer prefers to regard the forms as comprising a single species, varying enough in its chromogenesis and other biochemical features so that individual strains are often described as separate species. It is one of the most strongly proteolytic types in soil, liquefying gelatin so rapidly at temperatures of 20 to 22 C that one colony may well liquefy a whole plate of gelatin unless the temperature is kept at least as low as 18 C. Unlike *Bacillus cereus* and related spore-formers, *Pseudomonas fluorescens* can fluctuate very greatly in plate count, low on one day, high on the next, and absent entirely on the third day. This fluctuation, naturally, is due to its lack of spores or other resting stage. When its special nutrients are lacking, it must decrease rapidly to a mere minimum, and its numbers become too small to show on plates as highly diluted as must be employed to prevent overcrowding by other forms.

*Pseudomonas fluorescens* is quite easily distinguished on gelatin plates, although one must not look for its typical fluorescence under such circumstances. The identifying features will be discussed later.

#### *The Indigenous Flora*

In contrast to the zymogenous flora, the indigenous types are those that maintain fairly high and quite constant numbers, without showing appreciable increases or decreases according to presence or absence of special nutrients. Their exact function in soil is not fully understood; in fact they have often been neglected by students of soil bacteria. The present writer has always given much attention to them, chiefly because of the interesting speculation as to what rôle might be played by such numerous but unspectacular organisms. Although this interest began many years ago, it must be confessed that assigning a rôle to them is still largely speculative. Doing so is difficult for two reasons: their constant numbers make it impossible to correlate numerical fluctuations with definite activities in the soil; and when isolated and studied in pure culture, they prove to have so few positive biochemical characteristics that it is hard to assign any known chemical transformation to them. They seem to utilize practically the same nutrients as higher plants, e.g., nitrates and ammonium salts, and probably they maintain a low level of activity in soil, utilizing soluble forms of nitrogen as they are produced by the ammonifiers and nitrifiers. Conceivably therefore they may serve as rivals to higher plants, since they draw on the same sources of nitrogen; their rivalry cannot be serious, however, as bacteria are short lived, and are readily decomposed on death of the cells. It is even possible that their presence may be useful to plants in preventing the leaching out of soluble nitrogen when there are no plants to utilize it.

Their lack of strong fermentative reactions makes it difficult to classify them because they are so much alike physiologically. Furthermore, considering that,

as Lochhead and Taylor (29, 30) remark, they are "physiologically unstable", one has a problem of classification about as difficult as presented anywhere among bacteria. Almost the only progress that has been made in the way of classification has been on the basis of morphological features. On the basis of morphology we may consider four groups as follows.

*Actinomycetes.* As stated above, true actinomycetes (of the genus *Streptomyces*) may or may not be indigenous, and the writer prefers to regard them as probably zymogenous. Possibly some species may be zymogenous, the others indigenous; but since species differentiation is still too difficult to provide much idea as to the relative abundance of the different species under varying conditions, we cannot be sure. Their spores are almost universally present in large numbers, but there is good reason to believe that they become active only on special occasions.

*Arthrobacter species.* There are large numbers of forms present in soil which are intermediate between true bacteria and true actinomycetes; they are somewhat pleomorphic, showing rod-shaped, coccoid, and mycelioid forms. Jensen (24) called them species of *Corynebacterium*, Krassilnikov (26) of *Mycobacterium*. The writer has not accepted either of these proposals to place them in genera which are typically pathogenic, and originally named the most prominent species (in the soils studied) *Bacterium globiforme*. More recently in collaboration with Miss Dimmick (15) the proposal has been made to amend Fischer's name *Arthrobacter* (originally a *nomen nudum*, i.e., a genus without any species) to include these forms. These *Arthrobacter* forms resemble actinomycetes only in their occasional production of short mycelia; in type of growth they are like ordinary bacteria. They produce very small (punctiform) colonies on agar or gelatin media, and can be distinguished from the following organisms only by isolation and study to determine whether the typical morphological changes can be observed.

*Non-pleomorphic non-spore-forming rods (Agrobacterium).* There is a group of non-spore-forming rods in soil, essentially like *Arthrobacter* in physiology, but differing from it in showing no tendency toward branching or coccoid forms. They were originally thought to be close to *Alcaligenes*, because of their failure to produce acid or gas from sugars; but pointing out their difference, the writer (14) subsequently proposed the genus *Agrobacterium* to include them. The type species of this genus is *Agrobacterium tumefaciens*, a plant pathogen; and its best known non-pathogenic species is *Agrobacterium radiobacter* which shows much superficial similarity to the legume nodule bacteria (*Rhizobium*, spp.). Isolation of *Agrobacterium radiobacter* from soil usually requires special media; but there are on almost any plate from soil numerous colonies of bacteria that undoubtedly belong to the group, although they have not been given specific names, because of lack of positive characteristics on which to base specific distinctions. It is still uncertain whether few or many such species exist in the soil.

*Micrococci.* Mention should be made of this group here although the writer does not regard it as an important part of either the indigenous or zymogenous floras. It must be mentioned because Winogradsky, from microscopic observa-

tion of soil, concluded the "autochthonous" flora to be made up primarily of small cocci. The present writer, however, has found true cocci so rarely in soil as to be very doubtful whether those few that are found on the plates are of actual soil origin. Time and time again, however, cultures have been isolated from soil which seem to be micrococci when first examined; but after continued study it has been learned that their earliest stage is a rod form, and it has been concluded that they are actually *Arthrobacter* forms, the coccoid stage of which bears a striking resemblance to a pure culture of a micrococcus. After having this experience repeatedly, without ever observing a strain which is always coccoid in form, in young as well as in old culture, one naturally acquires considerable skepticism as to the occurrence of true micrococci among the indigenous bacteria of soil. Accordingly this group is mentioned here, merely to dismiss it from further consideration.

### *Methods of Identification*

One of the main objects of this paper is to assist students in soil bacteriology in identifying the members of the general soil flora which they cannot help but encounter if soil is plated on gelatin or agar media. The remaining section of this article deals with that subject. It must be recognized in advance, however, that the flora developing on such plates are only a part of the total soil flora, and may not comprise the most important bacteria. This statement is so true that the majority of soil bacteriologists regard plate counts of soil as of no real significance. Nevertheless, the plating of soil is still an interesting procedure, a preliminary step toward the isolation of pure cultures; and the study of such pure cultures in relation to soil activities may eventually solve soil problems that are still baffling. Accordingly it seems well to summarize the information now at hand which helps in identifying the organisms developing on such plates.

*Methods employed.* There are numerous media that may be used for plating soil when the object is isolation of members of the general flora; but two conditions must be maintained: little organic matter in the medium, and low temperature incubation. Peptone media are specially unsatisfactory, as peptone permits the overgrowth of spore-formers and proteolytic pseudomonads which prevent colonies of the more common but slowly growing bacteria from developing. The same unsatisfactory result occurs if incubation is carried on at temperatures higher than room temperature. If gelatin media are used, no nutrients should be added other than the salts normally present in tap-water, and even the tap-water may be replaced by distilled water without appreciably lowering the count. If agar is employed, nutrients must, of course, be added; but in addition to mineral salts it is well to include no more than 0.1% of glucose and a similar amount of some amino acid, ammonium salt, or nitrate. Various formulae for such agar media have been proposed, each having its own advocates among soil bacteriologists; apparently about the same flora develops on any of them, and apparently about the same counts are obtained, provided incubation is long enough and at a low enough temperature. Gelatin plates should be incubated at 18 C; agar at not over 25 C. Gelatin plates usually must be studied on about

the fifth to seventh day; agar plates may be incubated 10 to 14 days, and it is often desirable to do so, because of the slow growth of the most numerous soil bacteria.

When the medium is to be employed for the isolation of pure cultures for study, the writer prefers 12% gelatin, in tap or distilled water, pH 7.0. This medium is preferred because of the larger number of bacteria that may be recognized from their colonies. No one denies the obvious disadvantages of gelatin, chiefly arising from the danger of rapid liquefaction destroying a whole plate or at least the greater part of it. This liquefaction, however, is much less in tap-water or distilled water gelatin than in nutrient gelatin, and may be further minimized by incubation at 18 C. This low temperature is much easier to secure under modern methods of temperature control than it was formerly; when it is employed, and six plates (three of about 1/100,000 dilution and three of 1/200,000) are poured, it is rare that there are not enough satisfactory plates for study after five days, and incubation for seven days is often possible. The disadvantage of gelatin is outbalanced, in the writer's opinion, by the large number of types of colonies that can be distinguished in it.

The medium used for isolation may be almost any nutrient agar, as practically all the bacteria developing on any of the above mentioned plating media will grow on it. Isolation presents no difficulties except in the case of the numerous punctiform colonies in gelatin which sometimes fail to grow when fished by the inexperienced laboratory worker; for these organisms a useful technic is to employ a needle with a flattened point, and to pass the point around the colony once before the wire is completely cool, thus melting out a small block of the gelatin which can be lifted out bodily on the flat end of the wire and transferred to the agar slant. Other points of technic to be observed will become evident in the following directions.

*Classification of colonies.* Seven types of colonies can be recognized on gelatin plates:

- A. Large, liquefying, rhizoid to mycelioid. (Typical *B. mycoides* R colonies.)
- B. Rather large, liquefying, with a granular pellicle which often shows concentric structure. (Typical *B. cereus* R colonies).
- C. Rather small, liquefying, with a white flocculent center surrounded by a clear zone. (Colony of *B. megatherium* and the S forms of various spore-formers.)
- D. Small to very large, liquefying, structureless and quite clear, a single colony being capable of liquefying the entire plate if given the opportunity. (Typical *Pseudomonas fluorescens* colonies.)
- E. Under 3 mm diameter, non-liquefying, hard consistency, showing filamentous margin under low power of microscope; surrounded by a brown halo. (Certain *Streptomyces* colonies.)
- F. Like E but without the brown halo. (Certain *Streptomyces* colonies.)
- G. Punctiform, non-liquefying, of soft consistency, with entire margins as shown under low power of microscope. (*Arthrobacter* and *Agrobacterium* colonies.)

Of these seven types of colonies, the last three may include as much as 90% of the colonies on the plates. Types E and F are the actinomycetes (*Streptomyces*) colonies: type G, which cannot be distinguished from F without touching with a needle or examination under a microscope includes the *Arthrobacter* forms and the non-pleomorphic non-spore-formers (*Agrobacterium*) which make up a large percentage of the indigenous flora.

Occasionally other types of colonies may be encountered, conspicuous because of red, yellow, or orange chromogenesis. None of these is common enough to deserve special mention, however, except for an orange liquefying colony (often 10 to 20 mm in diameter) which, although usually absent, may sometimes be one of the most numerous colonies on the plate; the writer has identified it as probably *Pseudomonas caudatus*, originally described by Wright (52) in 1895.

Before going ahead with a key to the identity of organisms developing on such plates, one point must be emphasized. Bacteria growing on gelatin, or such agar media as those mentioned, are so numerous that, to prevent overcrowding of the plates, soil must be diluted 1 to 100,000 or more. It is obvious that bacteria occurring in numbers of 100, 1,000 or even 10,000 per gram are all but excluded from such plates, and if their colonies do appear they cannot be told from chance air contaminants; yet organisms occurring in the order of 1,000 per gram may well have an important rôle in soil activities. They are excluded from the present account not because of failure to realize their presence and possible significance, but because they are not encountered in the ordinary plating technic.

It is theoretically possible to obtain by plating methods any desired organism or group of organisms occurring in these smaller numbers in soil, by devising a special medium adapted to the bacteria in question but preventing the growth of the more abundant forms. For example, certain special plating media for *Agrobacterium radiobacter* have been devised (21, 36, 38) by which it is possible to secure this organism directly from soil, at dilutions of around 1/10,000. This organism also grows on ordinary media and presumably is responsible for an occasional punctiform colony appearing on them, but is easily overlooked; special media have been devised for it only because it is a particularly interesting species due to its close relation to the legume nodule organisms. Obviously, there must be many soil bacteria of similar frequency that have never attracted sufficient interest to have special media devised for them. All such organisms have to be omitted from the present survey of the field.

#### *Key to predominant groups and species*

##### Gelatin colonies rapidly liquefying

Colonies rhizoid or filamentous to naked eye.....R forms of *Bacillus mycoides*.

Colonies with granular pellicle which is often

concentrically ringed.....R forms of *Bacillus cereus*.

Colonies with a small floc of white granules at center. May be *B. megatherium*, or the S forms of *B. cereus* or *B. mycoides*. To distinguish between these three, inoculate into standard peptone agar slants.

Growth smooth, soft, with a tendency to become a dirty pink. Rods usually over 1  $\mu$  in diameter; spores about 1.2-1.5  $\mu$ ; chains of spores or sporangia rarely observed. ....*Bacillus megatherium*.



Growth colorless, smooth, soft, if remaining in the S phase; but wrinkled, membranous if reversion to R form has taken place (as often happens). Rods usually  $0.6-0.8\ \mu$  in diameter; sporangia swollen and usually remaining in chains for some time; spores about  $0.8-1.0\ \mu$ .....*B. cereus*.

Growth same, if remaining in S phase, but rhizoid if reversion to R form has occurred. Morphology exactly like *B. cereus*.....*B. mycoides*.

Note: Smith (39) insists that *B. mycoides* is only a variety of *B. cereus*. It is certainly true that their S phases are indistinguishable.

Colonies very large, if full growth has taken place, smooth and structureless, with only a minimum of cloudiness.....*Pseudomonas fluorescens*

Note: Fluorescence can usually be demonstrated by transferring to agar slants, especially if nitrate is present. Absence of fluorescence, however, is not a character of diagnostic importance.

Gelatin colonies small, with little or no liquefaction

Colonies varying in size from punctiform to about 3 mm, hard to the touch, with filamentous margins, as shown under low power of microscope; often surrounded by brown halo. (The larger colonies all easily recognized, but those too small to permit demonstration of the typical tough consistency require careful microscopic examination to be sure they belong here.).....*Streptomyces* spp.

Note: There may possibly be more recognizable species in this genus than in any other group of soil bacteria; yet species identification is difficult, and at present is attempted only by specialists of the group. Distinctions between species are based on: (a) certain morphological features which are difficult to describe precisely; (b) chromogenesis. Chromogenesis is the most striking feature, and is frequently of real diagnostic value; but it must be used with caution, because the pigments produced are ordinarily pH indicators, and to use them for species distinction one must either control the final pH, or at least take pH into account. Because of these difficulties no key to the species of this genus is given here, although there are 73 of them listed in the Sixth Edition of Bergey's Manual.

Colonies usually punctiform, practically never over 2 mm, soft, and with entire margins, as shown by low power of the microscope. These comprise partly simple non-spore-forming rods (*Agrobacterium*) and partly *Arthrobacter* types. To distinguish between them, slant cultures should be made on standard agar, and daily microscopic preparations made for 4 or 5 days. From the appearance of these the following two groups can be recognized:

Remaining continuously in rod form or sometimes oval in shape

*Agrobacterium* spp.

Note: The few definitely named species in this genus, *A. radiobacter* and certain plant pathogens, are not sufficiently abundant in soil to appear on ordinary plates. There are always, however, numerous *Agrobacterium* colonies of unnamed species; perhaps one, or two, or many species are represented.

Appearing as rods for 12 to 48 hours and then becoming spherical; large spherical bodies (termed cystites by Jensen) are found, and branching forms occur in liquid media. These *Arthrobacter* species may be distinguished from one another by the certain morphological features and by the presence or absence of yellow chromogenesis. The two species which the writer has found among the predominant soil forms are both non-chromogenic and are so regular in morphology after 3 or 4 days on ordinary agar as to appear like micrococci; whereas another well-known but apparently less common form, *A. helvolum*, is yellow and shows considerable morphological variation in such cultures. The two found commonly in soil by the writer may be distinguished as follows:

With diastatic action on starch, as shown by starch agar plates

*Arthrobacter globiforme*.

Showing no diastatic action on starch agar plates. . . . . *Arthrobacter simplum*

This key is very crude and perhaps over-simplified. It is not intended to permit the identification of every organism that may be found on plates from soil; in fact, it is not intended as a complete key to species of even the predominant types (as evident from the above "note" under *Streptomyces*). It is offered chiefly in order that a beginner in soil bacteriology may employ it to find his way into the field and to get some idea of the identity of the forms he is most likely to encounter.

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HERBERT W. CONN  
Photograph taken about 1915

## PROFESSOR HERBERT WILLIAM CONN AND THE FOUNDING OF THE SOCIETY<sup>1</sup>

H. J. CONN

*Geneva, New York*

In December, 1898—that is, just 50 years ago this coming winter—three members of the American Society of Naturalists, at a New York meeting of that Society, met together informally to discuss the question whether a society specially dedicated to bacteriology as a science was called for in this country. As a matter of fact, bacteriology as a science was a new idea in those days; it was chiefly thought of as an adjunct to pathology. The public had heard about “germs”, but if you told a layman that you were a bacteriologist, you would get a blank look in return, whereas if you had said chemist, botanist, or zoologist, there would have been some comprehension of your occupation. Of the three men in question, one was a general biologist, the other two pathologists. The chief question they discussed was whether an organization was needed which would appeal both to medical and non-medical men who were interested in bacteriology *as such*—not merely as a phase of pathology. These three men realized they were interested themselves in these broader aspects of bacteriology and felt that there might be others similarly minded in this country, enough eventually to form a society of perhaps 100 members.

The three men were A. C. Abbott of the University of Pennsylvania, E. O. Jordan of the University of Chicago, and my father, Prof. H. W. Conn of Wesleyan University. Of the three men, I naturally know the last-named best; and it is of him I wish to speak today.

I judge from comments of others, even more than from my own recollections, that he was in many respects an unusual man. He was an energetic organizer, yet suffering much of his life from what we now call an inferiority complex. He was an inspiring teacher, yet retiring in his ways and not at all gifted socially; in other words, he was unwilling to talk much unless he thoroughly knew his subject, and then he could be eloquent. He was an outstanding advocate of personal and public hygiene, although in his own ways he often failed to practice what he preached. He had force of character sufficient to put across those policies in which he was interested; yet he so hated argument that he would often get up and leave the room if conversation became argumentative. Although he was inspiring to his students, he was apparently so afraid of influencing his son's choice of a profession that he almost never mentioned bacteriology to me when I was a boy, and it was not till many years later that I realized he had wanted nothing more than for me to follow in his footsteps. He had a complex character, and I sometimes think that, seeing him in his daily life, I knew less of his character than his students who saw him only at class and in the laboratory.

<sup>1</sup> Amplification of Presidential Address delivered before the Society of American Bacteriologists at its Forty-eighth General Meeting, Minneapolis, Minn., May 12, 1948.

Years ago I was urged by one of his students to write his biography, and I replied that I did not know him well enough. Indeed, I have undertaken this subject for my address with some trepidation; the only reasons I now feel capable of handling it are that certain diaries and reminiscences of his, which had long been lost, came to light a few years ago, and in addition to reading them I have been having considerable correspondence of recent years with former students and associates of his.

Probably most of us are familiar with the famous story of Pasteur, telling how at a dinner he called attention to the importance of washing the germs off the skins of grapes before eating them, and then later absent-mindedly drank the water in which he had washed them. I do not think I ever knew my father to do that; but he would lecture repeatedly about the dangers of the common drinking cup—and then promptly use one himself the next time he wanted a drink while in a public place. He used to hate screens and screen-doors worse than flies, and my mother was forever having to shut them after him; so, when the connection of flies to typhoid was brought out, I think he was upset mostly because it gave my mother an unanswerable argument as to why he should not leave a screen door open. As a matter of fact he was somewhat fatalistic where matters of his own health were concerned, and many a recollection we members of the family still retain indicates that he regarded the rules of hygiene he taught as applying to others but not to himself.

But it was an entirely different attitude that made him a good teacher. It was partly because he had very human ways that endeared him to his students. Back in the days when college professors were supposed to be distant, dignified and immaculately dressed individuals, he was anything but that. Had he lived in this generation he would have delighted in the informal attire that is permissible so much of the time; but he did his best, for those days. His unconcern about the appearance of his clothes was a matter of much distress to my mother, but I do not think his students thought any the less of "Herbie" (as they used to call him, sometimes almost to his face) on that account. She was particularly mortified one morning when, after he had been gone an hour, one of his students came to our house; "Prof. Conn came to work this morning" he informed her "without his collar and necktie, and he sent me here to get them for him." I can imagine her feelings. With the stiff shirts of those days, the lack of a necktie alone would have been conspicuous; presumably too he had been to chapel, as the college day at Wesleyan always began with chapel services!

So the boys must have thought of him as a much more human person than the average professor. Also he could be inspiring. He might come into the laboratory some afternoon where three or four students were working, perch on one of the desks with one foot up under the other knee, and begin giving a very informal lecture. The boys would usually stop their work because they knew they were going to hear something interesting; and besides they suspected that in "Herbie's" courses less stress was laid on the amount of work done than on the student's interest in the work. Perhaps he might have just returned from some meeting, and, if so, he was always full of anecdotes about the men he had seen and points of

technic he had picked up from them. Almost invariably he would pick out of his pocket a small pad of paper with loose sheets torn off and attached to it by a rubber band. You see, he had the habit, whenever he heard anything that might specially interest someone, of writing that fact on a slip of paper from this pad under the name of the person to whom he intended to tell it. He kept the slip till he had done so, and then would throw it away. This pad with its attached slips became well known to his students and associates.

Another strong feature of his as a teacher was a way he had of driving a point home. In a lecture, he would pass rapidly over the details which he knew would be promptly forgotten even if his students took the trouble to learn them; but would stress and reiterate the fundamental points, even though they were so elementary many of his hearers knew them in advance. Most often he would repeat himself in different language to avoid monotony; but sometimes he would even repeat the words. Professor Buddington of Oberlin relates something about one of the lectures which he says amused him greatly. He was an associate of my father's for several years and used to sit at the back of the room through all the lectures so as to know where to take up a subject if he were suddenly called on to substitute for the regular lecturer. In one lecture on foods, the different values of proteins and carbohydrates had been discussed and he drove home his conclusions by coming out emphatically with the practical advice: "So gentlemen, remember, always eat *meat* with your *potatoes*! *Meat* with your *potatoes*!" Professor Buddington says he was much amused to see that every boy in sight of him, however little attention he had been paying, wrote in his notes "Eat meat with potatoes". Probably they had done that every day of their lives; but he had emphasized it in such a way they were unlikely to forget the underlying physiological principle.

This tendency to be repetitious undoubtedly helped him in his teaching; but in his writings it sometimes became tiresome, and my mother who edited most of his books, had to cut out a good deal of it. Occasionally it would even provoke a smile on the faces of his students, as when he would describe cocci as "little round, spherical balls".

By the time Professor Buddington was at Wesleyan, my father's courses had become very popular; he not only knew how to talk with students in an inspiring way, but he also had a somewhat original theory as to a teacher's function which was brought to my attention by two things he said to me while I was in college.

Once, for instance, I asked him why elementary physics was required of all but classical students; but no courses in biology. Didn't he think biology as important as physics? "I would not want any required course in biology," he answered. "You may notice that the elementary classes in biology are almost as large as if they were required subjects. I try to make them interesting enough so that most of the students will elect them. And I think they remember more of what they learn in my classes than they would if they *had* to take the courses." Some years later the faculty voted for a required course in public health, with Professor Conn as teacher. He began those lectures with some trepidation, I think, for he wondered if he could keep his audience interested in a required



course. He managed to; and he told me, with much delight, what rapt attention the faces registered and how he was usually kept 20 to 30 minutes after class answering questions by men who came up to the desk when the lecture was over.

Another time, I gave him a piece of information which I had picked up from my friends in college, and which I thought would surprise and shock him. "Do you know," I asked, "that your lecture courses in evolution and elementary bacteriology are selected by the men who are hunting for 'snap courses'?" "I don't doubt it" was his calm reply. "I make those two courses easy intentionally. I put into them ideas I think all students ought to know, and I want as many to take them as possible. Even though they take the courses just to avoid hard work, they learn something; and I think that does more good than it would if I made the work so difficult only the best students would elect the courses."

That, in a nutshell, was his theory of teaching. The elementary courses should be given by the best lecturer in the department and made attractive to everyone; the advanced courses, however, should be adapted to the best students only, and those electing them should be expected to do hard work.

I have tried so far to give some idea of his character; I do not think this the occasion to go into detail about his career. I do feel, however, that I should say a little about his early life to show how he became specially interested in bacteriology, and hence in founding this Society.

Although he seemed to be a healthy and very energetic man during the prime of his life, he had been a sickly boy—with what complaint no one knew; and considering that he finally died of heart failure, it is not impossible to conclude that his comparatively short life may have been due to the then unrecognized condition of rheumatic fever in his boyhood. However that may be, his early illness had quite an effect on his life because he was soon taken out of public school, and at the private school where he did go, he failed to get the contact with other boys that was more normal for one of his age. He liked school well enough, and the harder he had to work, the better; the one thing which bored him most at school was not having enough to do, since he could learn a lesson so much more quickly than any of the others. His greatest pleasure was reading. He read nearly everything he could get hold of in the small public library of his home town (Fitchburg, Massachusetts), and used to take such books to school to read during idle moments under cover of textbooks he was supposed to be studying but which were too elementary to interest him. This was back in the 70's (he was born in 1859) and not many good books for boys' reading were available.

The one subject he never could master was spelling, nor did he acquire that accomplishment to his dying day. Many years later in some reminiscences of his he wrote:

"I have often wondered why I was so unable to get my spelling lessons while all others were so simple and easy for me. . . . Arithmetic and Geography and even Grammar had some meaning and some reason for being, and the facts stuck in my mind. . . . But Spelling had no meaning whatever. The same letters had different sounds in different places and the same sounds had various fancy ways of being spelled . . . and my mind refused to hold such

a meaningless jumble as the illogical arrangement of letters. Nor has it ever consented to do this, for even to this day, after years of writing and proof-correcting, I am never sure of the spelling of an unusual word. . . . I well remember an embarrassing moment when writing on a blackboard an examination set of questions one of which contained the word *discuss*. Ordinarily I could have spelled it right, but not there in the presence of the class. I felt my cheeks turning red, and finally wrote it with a single *s* at the end and a peculiar little flourish which I hoped the class would take for a second *s* provided the word had two of them and would regard as a flourish if the word had only a single *s*. . . . I like to flatter myself that the reason why I never could make a good speller was that my mind was too logical to grasp such a nonsensical, unreasonable subject as the spelling of English words!"

It is, in fact, a little amusing in reading one of his journals about our family journeying in Europe to learn that we *rowed* along a quiet country *rode*, but on another occasion *road* a boat on a river. He seems to have used all three of these homonyms interchangeably.

He told me once that an important thing for any scientist was to learn to write well, "And I don't mean good hand writing or good spelling", he added. "You can get people to correct your spelling and to typewrite your manuscript. But you must know how to express ideas clearly."

He went to college at Boston University. That proved a good place for work, but with very little college life. Such a college suited him pretty well, but it did not help him in learning to become a good mixer. He joined a fraternity, but did not seem to have cared much for the associations that brought him. One of the experiences, however, which he did look back to in later life as of much help to him was an informal attempt at teaching. In anticipation of a difficult examination in physics, the whole class decided to get together, without any faculty member, and to study for the exam. He was voted chairman, and began asking questions of the others. He soon discovered that he had to answer most of the questions himself; and was not contented with answering them, for he felt that each one called for an explanation. As a result the class pronounced him a better teacher than their professor, and he began to think that perhaps teaching should be his career.

Certain events at Boston helped him decide on biology. One of these was his attendance on a series of lectures by Professor Farlow, not at the college, but at the Lowell Institute. His lectures were on Lower Forms of Life, and Father could never forget how he began his first lecture by uncorking a beer bottle, and remarking: "You have all heard of 'popping the question'; we will now proceed to 'question the pop'!" the young listener became so interested in these lectures that he considered seriously "questioning the pop" as his particular specialty in life—only remember, there was no such subject as bacteriology known in those days.

This fact is well illustrated by an anecdote he used to tell of something which took place a few years later (in the early '80's). He was then at Johns Hopkins, studying for his doctor's degree; and in later years used to recall how a tall, lanky young man appeared on the scene and announced he wanted to study bacteriology. According to Father, he was not given much encouragement, and was informed that only a genius like Pasteur could study bacteriology; so he went

elsewhere. Some years later, my father had a chance to meet one of America's most distinguished bacteriologists, and was surprised to recognize him as the young man who had been unable to find any bacteriology at Hopkins. He was Theobald Smith. Considerably after my father's death, I had a chance to tell this anecdote to Dr. Smith himself, and he was somewhat amused; his recollection of the matter was that he went to Hopkins to get one particular course, and when that course was over he left. Whichever version of the story is correct, it emphasizes the fact that the nearest any student could come in the 70's and early 80's to specializing in bacteriology was to major in general biology.

The young man we are most concerned with began taking biology in his junior year at Boston University, and after the first lecture he was interested enough to come back the following Saturday to look at microscopic animals under the microscope. This interest led to closer contacts with teachers in that field, and finally to an invitation to spend some time at a small marine laboratory that had been started privately by Professor Hyatt at Annisquam, Mass. This was the beginning of many summers that he spent at marine laboratories, most of them later on at Cold Spring Harbor where he was Director for several years.

His graduation, in 1881, was apparently more or less of an anticlimax to him. He graduated second in his class and felt that some recognition of that fact should be made in the graduating exercises. No attention was paid to him, however, and that rankled in his mind for most of his life. About 30 years later he was mentioning his college days in a letter to me, and added: "My graduating day was a very unhappy one, for I felt that I had not been treated fairly, and I have never had any interest in going back to college since." Undoubtedly this disappointment contributed to his inferiority complex; nevertheless, by this time his career had taken shape and from then on he followed it to a logical conclusion, and few if any of his associates ever suspected he suffered from a sense of inferiority.

The first real progress in his career came during his years of graduate study at Johns Hopkins where he was located from 1881 till summer in 1884. There he seems to have fallen into congenial surroundings. He majored under Dr. W. K. Brooks, one of the leading zoologists of the day, roomed at Dr. Brooks' residence in Baltimore, and seems to have made a good impression on him almost from the start. Dr. Brooks was in charge of the Chesapeake Zoological Laboratory which met some summers at Beaufort, N. C., and once or twice at Hampton, Va.; he took my father with him to this laboratory every summer while he was a student at Hopkins. He also had my father give four lectures a week and assist in laboratory instruction during his last year at Baltimore. The first publications (18 to 27) signed by H. W. Conn (dealing with invertebrate zoology) were the result of his work at Baltimore, Beaufort, and Hampton.

It was while at Hopkins that he became engaged to a girl who was his sister's best friend and whom he had known for a good number of years. When she died a few years ago I found in her possession what was probably the first letter he ever wrote her, dated at Boston soon after he started his college work there, and while she was at Smith College. It is written in formal style, copybook





handwriting, totally unlike his usual scrawl, and is interesting enough so that I trust I will not be violating the secrets of the departed by reading it:

"Julia:

I wish to find out whether there is a young lady in Smith College, by the name of Hattie Herrick, and thought that perhaps you would be as kind as to inform me. The lady to whom I refer was a schoolmate of mine at Ashburnham. She entered college last year and I wish to know for especial reasons whether she is there now and how she stands in her class. If it would not be too much trouble I would be greatly obliged to you if you would find out and let me know.

I am enjoying myself very much this term. I am engaged in studies in which I am especially interested and have tickets to courses of lectures upon these very same subjects. Don't you wish you lived in Boston, with a library of 250,000 volumes, 15 or 20 free lecture courses, and other advantages too numerous to mention?

Excuse the liberty I take in writing to you, for I wish to know about Miss Herrick very much.

Ever,

H. W. Conn"

I think it quite safe to assume that his real reason for writing this letter was *not* to get information about Miss Herrick! I also think it equally safe to guess that his real reason was attained; for Julia replied promptly enough so that he could answer her letter two weeks later (the second letter of his being still in existence); in this second letter he thanks her profusely for the information concerning Miss Herrick, but then goes on to other things. I presume their correspondence flourished after that, but no more letters are in existence except one which he wrote in 1882 (his second year at Hopkins) just after he had asked her to marry him. His diary mentions writing a letter of proposal, which is no longer in existence, nor do we have her reply; but I do have a lengthy letter of his written a little later showing that she had not yet given a definite answer. This letter I will not read. I will only remark that although he was presumably trying to add a few more words to win her over, he actually filled the letter mostly with comments about his own lack of prospects and his presumption in asking her to marry him. "The life of a scientist" he writes "is at best not enviable. It is sure to be filled with trials and sacrifices."

That may have been the way to win his particular girl, however; for they became engaged that fall, although they were not married until 1885, three years later.

From things he wrote in his diary those days, we know he was full of doubt as to whether he could succeed in life. How much of this was the natural feeling of any young man just beginning his career and how much was due to the sense of inferiority which had haunted him since his boyhood, I do not know. But I do know that he had another source of mortification, about which he was so sensitive that he did not confide it, except in barest hints, even to his diary: he was so youthful in appearance that he thought people laughed at him. In those days, when almost every college student encouraged a full beard, my father could hardly persuade more than a mere down to appear on his lip—even three years after graduation. After making up his mind to teach, he must have won-

dered if he could hold his own with students, most of whom would undoubtedly look older than he did. He succeeded apparently; but he had his moments of mortification—as for example when the expressman, who was moving his belongings into the apartment where he and my mother were to live right after they were married (when he was 26 years old), made some caustic remarks about college *freshmen* who brought wives to college with them!



Herbert W. Conn, 1886. Photograph taken during his second year at Wesleyan

He was then at Wesleyan University, where he spent the rest of his life. He had accepted a position there promptly on getting his doctor's degree in 1884; he was to be in the Department of Natural History, with the idea of heading a new department in Biology as soon as that could be arranged. He was thus the founder of the Biology Department at Wesleyan, and remained its head till he died in 1917. As I have implied before, he was a born teacher; and in spite of his numerous outside activities, he never neglected his college work, but built up a strong department and turned out numerous students who made themselves famous in their chosen fields. At first his chief interest was in evolution; but during the 90's his major attention came to be directed toward bacteriology, in

which he made his greatest reputation. He began teaching bacteriology around the turn of the century, and had a big lecture class in that subject; but his laboratory classes in that subject had to be kept small, largely from lack of equipment. His laboratory instruction in bacteriology was most informal. The student was first expected to make up his own media; then he was told to plate out water, milk, or whatever else he wanted, count the colonies, and pick up a number of



Herbert W. Conn, about 1905

pure cultures. Most of the rest of the course he was allowed to play around with those cultures and to see what he could find out about them. The scheme certainly took little of the professor's time and worked fine for the brilliant students; it was not so effective in case of the less brilliant men, but my father tried to weed them out of his laboratory classes before they had gone far enough to begin bacteriology.

His outside activities during this period were almost too numerous to mention: He was Director for several years (1891 to 1897) of the marine Biological Laboratory at Cold Spring Harbor; he wrote 22 books, many of which went through several editions; he was in charge of bacteriological research at the Storrs Ex-



periment Station from about 1890 till 1906 and taught a class in bacteriology at the Agricultural College there (now University of Connecticut); he was very active in public health work, having a lot to do with the founding of the Laboratory Section of the American Public Health Association, and serving for over ten years (1906-1917) as Director of the Connecticut State Board of Health Laboratory; he was a popular lecturer in scientific fields, making lecture trips each year of quite an extended nature; he was for some 20 years superintendent of a Sunday School in Middletown; and lastly, as we all know, he was one of the three founders of this Society.

His growing interest in bacteriology during this period, of course, most concerns us. His first outstanding work in that line was in the field of dairying. When he began studying bacteria in milk, no one realized the great importance of the lactic fermentation in milk; and it was his work, with Esten, at Wesleyan, (37-46, 49, 52, 56-60, 63-67, 71, 84, 85, 89, 92, 97) that showed how the lactic acid bacteria predominated over all other types initially present. This led to various practical applications in the ripening of butter and cheese as well as in preventing spoilage of fluid milk. It is hard for us to realize today what a revolution there was, between 1900 and 1920, in the methods of milk production and distribution, during which period the change took place from the unsanitary, rapidly souring milk of the last century, to the relatively safe milk of good keeping quality which we know today. A large contribution to this revolution came from the research of the Wesleyan investigators in the 90's—followed up by the stress on sanitary control in which Professor Conn took a leading part after 1900 (62, 83, 86, 87, 89-92, 101-103).

Another important activity of his in the 90's (which in fact continued till his dying day) was in giving popular lectures. In the days before movies and radio, entertaining lecturers were in more demand than they are today; and when any member of a faculty showed talent in that line his college used to be glad to send him on lecture trips for the sake of the advertisement which it gave the institution. Wesleyan had several gifted lecturers on its faculty, some in classical and literary fields, some in the sciences; Professor Conn used to be considered one of the best of their scientific lecturers. It was for this reason that he needed to have a substitute (like Professor Buddington) present in his classes to supply for him when he was suddenly called out of town. He knew how to present a subject in an interesting way; and he also had the rare gift of knowing how to be brief. He told me, in fact, how on a trip to Schenectady he had been asked to address about 700 scholars at a high school and planned to give one of his stock lectures on bacteria which ordinarily took an hour or more but *could* be crowded into 45 minutes. Just before beginning he asked the principal how long he could have and was told *15 minutes*. He told me later it made him "hot for a minute" to think how to get 45 minutes crowded into 15. He added: "Well, I did it, and did not take more than the time suggested; judging by the tremendous applause that followed I judge I did it all right."

He seems to have driven himself hard all his life. Social life did not appeal to him and he avoided it as much as he could. His chief way of relaxing was to





turn from one form of work to another. His outside interests were many, but they were nothing that could truly be called hobbies; when he really became enthusiastic over one of them, it later became a main part of his life work. That was the way he worked into bacteriology.

His interest in bacteriology was given a tremendous impetus in 1894 when there was an outbreak of typhoid at Wesleyan, which he was successful in attributing to oysters fattened under unsanitary conditions (55). This was the first time oysters had been blamed for typhoid; and the logic he used in eliminating all other sources of infection and in definitely incriminating the oysters from one particular source made a great impression at the time in the scientific world. It not only added to his reputation, but firmly established his interest in bacteriology. It led in later years to activities in the public health field, although at first his chief interest was in dairy bacteriology.

Further stimulation was given to his interest in bacteriology by a trip to Europe he made in 1897-8, when he was granted a leave of absence from Wesleyan. I was 12 years old at the time, and remember the trip well, although I was naturally too young to understand what his chief interests were in taking the trip. I can see now, however, that he went as much as anything to take courses under distinguished biologists and to meet others in that field, but that he shortly became most interested in his contacts with German, Danish, and Swiss bacteriologists—Koch, Ostertag, Bang, Freudenreich (see 70). His trip was equally worth while to him in other ways, as he was a keen observer and much interested in the ways and characteristics of the people among whom he traveled. He wrote a day-by-day journal of the trip in which he recorded not only his doings but his impressions of the people and things he had seen; to me it is one of the most interesting things he ever wrote, more so than any of his published books. One of his comments on the German character, written nearly 20 years before the first World War is so prophetic I want to quote it, although it has nothing to do with bacteriology:

"The German makes a first-class citizen. He is willing to obey without knowing any reason for it except that it is the law. With good leaders the stolid, phlegmatic, unimaginative German can be led to almost anything, and they thus make the very best soldiers when things are all planned and go as planned. Their wars have been successful, since they have been carried out as planned. But I have an idea that if they met with reverses the whole army would lose its head and be utterly unable to readjust itself to any new condition. All this is surmise, however, but their slowness in grasping an idea makes one wonder what they could do in an emergency. As long as things go as they plan they can be relied upon, but when they once get their plans upset, then—good-by to their great power—this is my opinion."

I do not know whether or not the idea of a society of American bacteriologists occurred to him on this European trip. It is not impossible that something may have been said, in his conversations with European bacteriologists, to put in his head the idea that America needed an organization dealing with bacteriology as a science. At all events it was only a few months after his return—December, 1898—when he had the historic conference at New York with Abbott and Jordan,

and one of the three broached the idea which led to the formation of this Society. Apparently, at that meeting, Dr. F. P. Mall, then Professor of Anatomy at Johns Hopkins, referred to either Dr. Jordan or Dr. Abbott as a "lost soul", because there was no existing section of the Association devoted to his interests, and someone suggested that the bacteriologists ought to organize a section of their own.<sup>2</sup> Jordan and Abbott thought the idea worth while; and the former immediately set out to find Conn. They proposed the formation not of a new section of the A.A.A.S., but of a distinctly new society. They decided, according to a statement they published a few years later that there was no society "whose nature was such as to bring together the large and growing number of investigators who are studying bacteriological topics. It was felt that the rapid development of this subject along biological, agricultural, industrial, as well as hygienic and pathological lines, is creating a special branch of science; and it was believed that an association of investigators in these various lines would be mutually helpful."

They corresponded about the plan during 1899 (See Appendix, p. 291), and I imagine the idea was broached informally to various friends of theirs during the

<sup>2</sup> In a letter written June 8, 1922, Dr. Abbott recorded his recollections of the founding of the Society in these words:

"At the 1899 meeting of the 'American Association for the Advancement of Science', held at Yale University, New Haven, Connecticut, Mall (the late F. P. Mall, Prof. of Anatomy, Johns Hopkins University) and I had been lunching together when Jordan (F. O. Jordan) joined us while we were having an after luncheon smoke. Mall exclaimed—'here's a lost soul', or 'an unattached wanderer' or some equivalent expression, meaning that Jordan and a number of others like him, did not find themselves at home in any of the existing sections of the Association; and there was no apparent way for this group of men to become active in the association without the organization of a new section or Society to which the bacteriological contributions could appropriately be made. He said he and Jordan had been talking the matter over and asked me if I would cooperate with certain other interested men and organize a special society into which bacteriologists as such, could be admitted and be encouraged to develop the phases of the science that made the most appeal to them.

I gladly embrace the opportunity to be of what seemed to me real service and soon found myself one of a self-appointed Committee of three, (Jordan and the late Prof. Conn of Middleton, Conn., being the other two), who took it upon itself to call a meeting of those men in the country who were regarded as most likely to look favorably upon the project and become active workers for its success."

Some time latter this comment was shown to Dr. Jordan, and in a letter of June 24, 1935, he wrote as follows to the Society's Archivist:

"My recollections of the first conversations with Abbott and Conn are a little different from those of Professor Abbott. Let me give them here.

Our first conversation took place at the meeting of the Society of American Naturalists in New York in 1898 (I think the enclosed correspondence bears this out). I was coming down the steps of some building where meetings were being held when I fell in with Mall, whom I had known well both at Clark and Chicago, and stopped to chat with him. He said, 'Why don't you bacteriologists have a Society of your own? I just met Abbott also wandering around like a lost soul.' I suppose the meeting at luncheon to which Abbott refers must have taken place a little later because I distinctly recall talking with Abbott after seeing Mall and then going with him to look up Conn."

year, but no formal step was taken until October when a letter was sent out to about 40 American bacteriologists, which ran as follows:

My Dear Sir:

An attempt is being made to organize a society of American Bacteriologists upon the lines followed by the Society of American Physiologists, the Society of American Morphologists, and the kindred associations that meet yearly as "affiliated societies", with the Society of American Naturalists. It is thought that such an association will conduce to unification of methods and aims, will emphasize the position of bacteriology as one of the biological sciences, and will bring together workers interested in the various branches into which bacteriology is now ramifying.

It is hoped that you will be willing to aid in this undertaking. Should you be willing to cooperate in the formation of such a society, will you kindly communicate with Prof. H. W. Conn, Wesleyan University, Middletown, Connecticut, before November 1st.

The first meeting will be held with the affiliated societies at New Haven, Connecticut, during the Christmas vacation. You are requested to send the titles of papers to be presented to Prof. A. C. Abbott, Hygienic Laboratory, University of Pennsylvania, Philadelphia, Pa. Titles of papers should be in Professor Abbott's hands not later than December 10th. A program giving details as to time and place of meeting, titles of papers, etc., will be issued later.

A. C. ABBOTT

H. W. CONN

E. O. JORDAN

This letter, I think, was neither duplicated nor mimeographed (those processes being hardly perfected in those days) but was either handwritten or done individually on the typewriter; neither did college professors have stenographers at their call at that early time. Because of pressure of other duties, Dr. Abbott did not send out any of these letters; the other two members of the self-appointed committee apparently apportioned the job between them. I definitely remember quite a number of those letters going out from our house. The replies, as directed, came to my father, and in many cases there was follow-up correspondence. According to the statement published by the three men later, "The responses to this letter were immediate and emphatic. With the exception of three, all to whom the letter was sent responded and in every case was expressed a feeling that the organization of such a society was not only feasible, but eminently desirable."

The meeting for organizing was held at 2 P.M., December 28, 1899, at the Medical School Building of Yale University, with 30 in attendance. Prof. W. T. Sedgwick of the Massachusetts Institute of Technology was invited to take the chair, and he presided at all the sessions that year. Dr. Abbott had prepared a program, of which seventeen papers were presented at this meeting; including one by H. W. Conn on "Natural varieties of bacteria"; eight other papers were listed on the program but not presented. At the third session, December 29th, the committee to draw up a constitution reported and the first constitution of the Society (perhaps 200 words long) was adopted; Dr. Sedgwick was elected President; Dr. Abbott, Vice-President; and Dr. Conn, Secretary-Treasurer. It was decided that the original membership should be those responding to the

letter of October 1899 and any others who had attended the first meeting. Thus computed, there were 59 original members.

The New Haven members of our Society have long taken pride in being located at the birthplace of our Society. A 30th anniversary meeting was held there in 1929, and a 40th in 1939, and hopes were expressed that the 50th anniversary would also be held there. It is a matter of much regret to the New Haven members that the Society has now outgrown the facilities afforded by their city, so that we cannot meet there next year. The best we can do is to meet in Baltimore in 1950, just 50 years after the *second* meeting of the Society was held in *that* city.

At the original New Haven meeting it was decided to limit active membership to 100; but the Society grew much faster, even in those days, than its founders had thought possible, and it proved necessary to raise the limit occasionally to avoid a long waiting list. When my own application for membership came up in 1910, the limit was either 200 or 250, and there was just one vacancy. There was another name besides mine being considered for membership; and to avoid having to choose between us two, the Council made Father an honorary member, thus creating one more vacancy on the list of active members. After that it was a joke of his that they dropped him from the active list to put me on. Of course, a few years later, the Society removed all limit to the membership, the better to support the Journal of Bacteriology, and we all know how rapid its growth has been since. If my father, in 1898, when he first conceived the idea of the Society, could have pictured this meeting, 50 years later, at which is son is presiding, with a registration of about 1000, and a membership of about 3,500, he would have been decidedly surprised!

But to come back to the early days. The membership dues were \$1.00 a year, and the Secretary-Treasurer was allowed to use the *tremendous* sum thus realized for clerical help. It would not have gone far toward hiring a stenographer, but I believe he hired my mother to type his form letters to the members. He held that office till the 1901 meeting at Chicago (the 2nd meeting of the Society having been held in Baltimore in 1900); at Chicago he was elected President, and Jordan became Secretary. His influence in the Society continued for many years after he was out of office, however, and he was a perennial member of the nominating committee. Officers were elected quite informally in those days: a nominating committee was appointed by the President at the first session, which reported at a later session, with a single candidate nominated for each office. This put so much responsibility on that committee that serving on it became embarrassing to my father eventually, and I can remember how in his last few years (1914 to 1917) he used to stay away from the first session so that he could not be put on that committee. He lived just long enough to see the Journal of Bacteriology started and the membership limit removed, but not to see the Society begin its rapid growth in members.

Such were his activities, or at least all I know of them, in connection with this Society in its early days. To round up this brief account of his life, however, I should say something about the nature of his activities during his last 15 years.

The only one of his earlier activities which he gave up at this time was his connection with the College and Experiment Station at Storrs. He gave this up at about the time he became director of the Board of Health Laboratory; so the result was a decided shifting of his chief interests from the agricultural to the public health field. This Board of Health laboratory was somewhat unique at its time. About the only practical methods of laboratory diagnosis then known were for diphtheria, pulmonary tuberculosis, and typhoid, and facilities for making such tests were available only in large cities. Connecticut was one of the first states to realize that the telephone and more rapid mail service made it possible to extend such a service to small communities located some distance apart. It was decided that Connecticut was small enough in area but sufficiently densely populated to make statewide service of the sort practical. The venture proved a success, and directing that laboratory occupied much of my father's time for the rest of his life.

It also brought him into closer contact with other workers in the public health field including Dr. C.-E. A. Winslow and Dr. C. E. North, who are still with us, and brought about very close relationships with Dr. William H. Park of New York City. One of his first dealings with Dr. Park in this matter was in 1900 when he and Dr. North made a visit to the laboratory where Dr. Park, with one assistant (Dr. Anna L. Williams, I believe) was undertaking for the first time to make bacterial tests of milk. They found him installed in one small room over a horse stable in East 15th St., not even knowing how to make the tests required. My father showed him on that occasion how to make plate counts on ordinary gelatin and on lactose litmus gelatin—the latter medium being one of my father's own devising and which he had used as the basis of his important work on the milk flora.

That was how the outstanding work of Dr. Park's Board of Health Laboratory in New York City started—with very humble beginnings. It was also the beginning of the association of these three men in their fight for the sanitary control of milk. They conceived the idea of sanitary milk standards, and pushed the idea for all they were worth (See 101, 102, 103). By the time my father died in 1917, the battle was practically won, but it had been a hard fight. For a man who always hated a fight, and had started out in life with an inferiority complex, he certainly obtained an amazing reputation as a fighter in those days—for there were strongly entrenched interests in the dairy industry then who were not at all anxious to see such standards enforced, and who managed to secure other prominent bacteriologists to fight their battles for them. It required many a hard-fought battle at meetings of the A.P.H.A., in legislative bodies, and at court trials, before such standards were generally adopted and enforced. The men who were associated with my father in these activities have been amazed when I told them of his inherent timidity and hatred for argument; they insist he kept those qualities well hidden from them!

I have nearly finished my subject; but I would like to say a few words of a more personal nature—concerning my own relations with him. In some ways I never felt as close to him as I might have been—as my only sister was, in fact;



but there was never any serious misunderstanding between us. Quite the contrary, I think he handled very well the problem I posed for him. It must have been quite a blow to him when he realized that his son, whom he knew to have certain qualities that made for success, and on whom he had undoubtedly been pinning hopes for the future, was becoming hard of hearing. Yet he never once made the mistake of showing undue sympathy or of letting me assume that success would be impossible because of the handicap. On the other hand he encouraged me to take advantage of any other sense or facility I possessed to overcome the difficulties I faced. I think it was largely due to him that I avoided the error so common among deaf people of becoming over-sensitive, and at the same time assumed from the beginning that the handicap was not going to keep me from success in life. On looking back now, I think I owe him more gratitude for instilling that attitude in me than for the indirect way he steered me into bacteriology—although I also appreciate the latter.

Perhaps I cannot end this account better than by quoting my sister. As I have already remarked, she was closer to him than I was in many ways; and long after he died, she wrote to me in reminiscence about him:

"Although he did not often speak of intimate things, what he said once he meant always. He never spoke until he was sure, and then not again. Indeed like most New Englanders he was blunt and not given to flattery. Like them he did not want to begin saying 'nice things', for as he explained, he would then have to keep saying them. If he liked something or someone and said so, he meant it, and there was no need of repeating.

His odd ways, you say, sometimes bothered you; while they tickled and delighted me. His indifference to so much that others held as important; his impatience with 'small talk'; his equal impatience with fancy desserts ('sweetened nothings' he called them; the only desserts for him were apple pie, mince pie and squash pie); his love of hearing rain on the roof; his horror of being late or of being kept waiting himself; his quick hurt at being interrupted or not listened to while he was talking; his dislike of dressing up to go out for the evening—these and many other traits gave him a peculiar charm to my eyes as I began growing up."

That sums him up better than I could in my own words; and all I need add relates to his passing on. In 1909 or 1910 (when he was about 50) he first realized that his heart was weak and that he must cut down on physical activity. Doing so was a hardship to him as he had always been energetic and so very active mentally as to be most unhappy when doing nothing. To stay at home because he had no way of going anywhere except walking, and to be dependent on taxis or other conveyances for local transportation on all his trips was very annoying to him. Even so he kept going, and a little before he died he wrote me a letter telling about his numerous engagements on a trip he had just taken which made me feel tired when I re-read it recently. I suspect such activities tired him also.

His death came suddenly, although we had known for years it might come at any time. He died on April 18, 1917, the very day that my daughter (who is now a member of this Society) was born. The suddenness was hard on those he left behind; but I am sure he would have had it that way could he have chosen. I cannot think of a better epitaph for him than one remark he made in the letter I just mentioned: "I don't seem to be put on the shelf yet."

## APPENDIX

CORRESPONDENCE RELATING TO THE FOUNDING OF THE  
SOCIETY OF AMERICAN BACTERIOLOGISTS

Between

H. W. CONN, E. O. JORDAN and A. C. ABBOTT

Chicago, Ill., April 6th, 1899.

My dear Prof. Abbott:—

I enclose a list of names as a starting-point for our proposed Bacteriological Society. Doubtless names that should be added to the list will occur to you and perhaps some names that I have included should be stricken out. After we have once organized other members,—and I think there are many desirable ones,—could be chosen.

I would suggest that a circular letter be sent to the men we finally decide upon as most available in this connection, and I will write out a rough draft of what I have in mind and send it to you for revision in a few days. I have sent a copy of the list to Prof. Conn, asking his opinion in regard to desirable additions and subtractions. Should we send out our circular letter this Spring or wait until October?

Very truly yours,  
EDWIN O. JORDAN

Middletown, Conn., Sept. 27, 1899.

Mr. E. O. Jordan,

Dear Sir:

I have waited about returning the enclosed list, thinking that the best time to send out the circular letter would be October. I have added a few names to the list, but have not thought wise to omit any.

It seems to me that you have included only desirable men in your list, and that all should be invited to join the proposed section of the Naturalists' Society.

I think that a circular letter should be sent out now as soon as possible inviting men not only to join, but to present papers at the meeting to be held in New Haven next Christmas. I hope that Prof. Welch can be prevailed upon to act as President for the first year, at least.

Let us now push the matter as much as we can for a month or two, and I feel confident that the section will then be a success.

I should be very glad to see a rough draft of the circular letter, as you suggest, although I have perfect confidence in your ability without submitting it to me for revision.

Trusting you can now push matters to a successful issue,

I am, respectfully,  
H. W. CONN

Chicago, Illinois, October 9th, 1899.

My dear Prof. Abbott:—

I enclose a draft of a circular letter concerning the formation of our proposed Bacteriological Society. I have also sent a copy to Prof. Conn and he will have it sent out to the gentlemen named on our list. If you wish to add any names to the list, will you kindly forward them to Prof. Conn. Both Prof. Conn and myself have added a few since the list was sent you last Spring, but there may still be some that we have overlooked. Would you be willing to undertake what I have suggested in the circular letter—namely, the arrangement of the program for the New Haven meeting? Prof. Conn, who is near New Haven, will arrange details as to time and place of meeting.

Would it be possible for you to see Prof. Welch and gain his consent to preside at our meeting of organization? If so, will you kindly communicate with Prof. Conn, in order that some statement to that effect may be inserted in the circular letter.

Trusting that we may now push matters to a successful issue,

I am,

Very truly yours,

EDWIN O. JORDAN

Philadelphia, Oct. 12th, 1899.

Prof. E. O. Jordan,  
Chicago University,  
Chicago, Ill.

My dear Prof. Jordan:

I must confess that your letter received this morning brings me face to face with a very serious matter,—namely, the organization by us of the new Society. During our conversation in New York last winter I agreed to support it, and shall keep my promise, but I say frankly that I have so many irons in the fire that I do not know at this moment how much support I can give it. At all events, if you think I can be of assistance in arranging the program I will be very glad to undertake it, but I must look to you and to Prof. Conn and to every one else interested in the Society to aid me in getting up a decent program for the first meeting. I have just written to Prof. Welch, asking him to preside at the meeting of organization, and as soon as I hear from him I will let you know concerning the matter.

Very truly yours,

A. C. ABBOTT

Middletown, Conn., Oct. 18, 1899.

My dear Prof. Jordan:

Your letter is received and I am glad to undertake my share of the work. I have prepared a letter similar you sent, with some additions, and have already sent a copy to each person in the list of addresses.

I will let you know later what responses I receive.

Yours truly,  
H. W. CONN  
(J. M. C.)

## PUBLICATIONS BY H. W. CONN

### Arranged Chronologically

#### I. Books

1. CONN, H. W. 1886 *Evolution of Today*. G. P. Putnam's Sons, New York.
2. — 1891 *The Living World*. G. P. Putnam's Sons, New York.
3. — 1897 *The Story of Germ Life*. D. Appleton and Co., New York;  
in *Spanish*, 1902, as *Nociones de Microbiologia* (Same publisher);  
in *England*, 1909, as *Germ Life*. Hodder and Stoughton, London.
4. — 1899 *The Story of the Living Machine*. D. Appleton and Co., New York;  
1915 *2nd edition*;  
in *Spanish*, 1901, as *Nociones de Biologia* (Same publisher);  
in *England*, 1912, as *Story of Life's Mechanism*. Hodder and Stoughton, London.
5. — 1900 *The Method of Evolution*. Silver, Burdette and Co., New York.
6. — 1901 *Agricultural Bacteriology*. P. Blakiston's Son and Co., Philadelphia;  
1909 *2nd edition*.
7. — 1903 *Bacteria, Yeasts and Molds in the Home*. Ginn and Co., Boston; 1912,  
*2nd edition*; revised 1917.
8. — 1903 *An Elementary Physiology and Hygiene for use in Schools*. Silver,  
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in *Spanish*, 1913, as *Nociones de Physiologia e Hygiene* (Same publisher).
9. — 1903 *Bacteria in Milk and its Products*. P. Blakiston's Son and Co., Philadelphia.
10. — 1904 *Evolution of Today*. G. P. Putnam's Sons, New York.
11. — 1904 *Introduction to Physiology and Hygiene for use in Primary Grades*.  
Silver, Burdette and Co., New York.
12. — 1907 *Practical Dairy Bacteriology*. Orange Judd Publ. Co., New York.
13. — 1908 *Introduction to Physiology and Hygiene for use in Intermediate Grades*.  
Silver, Burdette and Co., New York.
14. — 1909 *Advanced Physiology and Hygiene for use in Secondary Schools*. Silver,  
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15. — 1913 *Elementary Physiology and Hygiene for use in the Upper Grammar  
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16. — 1914 *Social Heredity and Social Evolution*. Abington Press, New York.
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York.

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18. CONN, H. W. 1882 *Development of Tubularia cristata*. Johns Hopkins Univ. Circs.  
1, 246.
19. — 1883 *Evolution of the decapod zoea*. Science, 3, 513.
20. — AND BEYER, H. G. 1883 *The nervous system of Porpita*. Johns Hopkins Univ.  
Studies of Biol. Lab., 2, 433-445.
21. CONN, H. W. 1883 *On radial and bilateral symmetry in animals*. Johns Hopkins  
Univ. Circs., 2, 73-74.
22. — 1883 *An instance of sexual color variation in Crustacea*. Id., 3, 5.
23. — 1884 *Evidence of a protozoa stage in crab development*. Id., 3, 41.

24. CONN, H. W. 1884 The significance of the larval skin in Decapods. Johns Hopkins Univ., Studies Biol. Lab., **3**, 1-26.
25. — 1884 Marine larvae and their relation to adults. *Id.* **3**, 165-192.
26. — 1884 Life history of *Thalassema*. (*Doctor's thesis*). *Id.* **3**, 351-401.
27. — 1884 Method of formation of trochosphere in *Serpula*. Johns Hopkins Univ. *Circs.*, **4**, 15-16.
28. — 1886 The limits of organic evolution. *Am. Naturalist*, **20**, 413-422.
29. — 1887 Scientific fact and scientific inference. *Id.*, **21**, 791-799.
30. — 1888 Coleopterous larvae and their relations to adults. Boston Soc. Natural Hist., **24**, 42-45.
31. — 1888 The germ theory as a subject of education. *Science*, **11**, 5-6.
32. — 1888 Bacteriology in our medical schools. *Id.*, **11**, 123-126.
33. — 1888 The significance of 'variety' and 'species'. *Id.*, **11**, 253-254.
34. — 1888 Germ diseases. *New Princeton Rev.*, **5**, 141-144.
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36. — 1889 Report of Prof. H. W. Conn. Bd. Water Commiss., Middletown, Conn., 24th Ann. Rept., 11-22.
37. — 1889 The bacteria of milk. Conn. Bd. Agr., 23rd Ann. Rept. 180-195.
38. — 1889 Bacteria in milk, cream and butter. Storrs Agr. Expt. Sta., 2nd Ann. Rept., 52-67.
39. — 1889 Bacteria in milk and its products. Storrs Agr. Expt. Sta., Bull. **4**, 2-12.
40. — 1890 The fermentations of milk and their prevention. Conn. Bd. Agr., 24th Ann. Rept., 228-244.
41. — 1890 Ripening of cream. Storrs Agr. Expt. Sta., 3rd Ann. Rept., 136-157.
42. — 1890 A micrococcus of bitter milk. *Id.*, 158-161.
43. — 1891 Some uses of bacteria. Conn. Bd. Agr., 25th Ann. Rept., 92-191.
44. — 1891 Bacteria in the dairy. Storrs Agr. Expt. Sta., 4th Ann. Rept., 172.
45. — 1891 The fermentations of milk and their prevention. *Science*, **17**, 272-277.
46. — 1892 Bacteria in our dairy products. *Pop. Sci. Monthly*, **40**, 763-774.
47. — 1892 Some uses of bacteria. *Science*, **19**, 258-263.
48. — 1892 Report of the summer school of the Brooklyn Institute for the season just closed. *Id.*, **20**, 157-159.
49. CONN, H. W. 1892 The fermentations of milk. U. S. Dept. Agr., Off. Expt. Sta., Bull., **9**, 7-75.
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52. — 1893 The ripening of cream by artificial cultures of bacteria. Storrs Agr. Expt. Sta., 6th Ann. Rept., 43.
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56. — 1894 The ripening of cream by artificial cultures. Storrs Agr. Expt. Sta., Bull., **12**, 1-20.
57. — 1894 Experiments in ripening cream with *Bacillus* No. 41. Storrs Agr. Expt. Sta., 7th Ann. Rept., 57-68.
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60. — 1895 Outline of dairy bacteriology by H. L. Russell; a review. *Science*, n.s., **1**, 189.
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63. — 1895 A year's experience with *Bacillus* No. 41 in general dairying. Storrs Agr. Expt. Sta., 8th Ann. Rept., 17-40.
64. — 1896 Experiments in cream ripening. Storrs Agr. Expt. Sta., Bull., 16, 3-16.
65. — 1896 Further experiments in cream ripening: flavor, aroma, acid. Storrs Agr. Expt. Sta., 9th Ann. Rept., 17-43.
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71. — 1899 Classification of dairy bacteria. Storrs Agr. Expt. Sta., 12th Ann. Rept., 13-68.
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76. — 1900 The ripening of cream. Storrs Agr. Expt. Sta., Bull., 21, 5-24.
77. — , and ESTEN, W. M. 1901 Le développement comparatif des différentes espèces microbiennes dans le lait. Rev. gen. lait, 1, 121-126.
78. — 1901 The Storrs Agricultural Experiment Station and its work in dairying. Conn. Bd. Agr., 35th Ann. Rept., 249.
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## CORN STEEP LIQUOR IN MICROBIOLOGY

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The publicity given to the development of the penicillin industry also has called attention to the value of corn steep liquor as a source of nutrients for microorganisms. Although considerable information on the properties of corn steep liquor has been accumulated, attempts to integrate this information have been rare (cf. 38). An effort will therefore be made in this review to describe the production and properties of corn steep liquor, and to evaluate its usefulness in microbiology.

### *Production of corn steep liquor*

Since corn steep liquor is a by-product of the corn wet-milling industry it would be insufficient to discuss its manufacture apart from the whole process in which corn, after having been shelled and air-cleaned, is soaked, and then fractionated into its principal components by a combination of flotation and wet-screening procedures.

To avoid losses of raw material and to keep sewage disposal problems to a minimum, practically complete recovery of the solids is desired. This is accomplished by the so-called "bottled-up" process whereby water is reused in a counter-current flow with respect to the corn and losses of the solids are kept to less than 0.5% of the dry substance of the corn. The technology of this process is discussed in detail by Kerr (26). A popularized but authentic description can also be found in a publication by the Corn Industries Research Foundation (7). For a discussion of the water balance and sewage disposal problems see Greenfield, Cornell, and Hatfield (20).

The corn is first soaked, or steeped in open wooden tanks at 45 to 52 C for 40 to 48 hours. Five to seven gallons of water are required for every bushel of corn. The water used in steeping is process water that has been used previously in other phases of the process, for example, the overflow from the gluten settling tank. During steeping the soluble materials are dissolved, the corn is softened, and its structure weakened and broken, which facilitates the grinding and further separations of its components. Just before the process water enters the tanks, SO<sub>2</sub> is added to prevent putrefaction and to assist in the extraction of the soluble compounds. The concentration of SO<sub>2</sub> is initially from 0.1 to 0.2%, but since most of the SO<sub>2</sub> is absorbed by the corn, it is lowered to 0.05% five hours after addition, and to 0.01% within ten hours. Moving in a general counter-current fashion, the most dilute water is placed on corn that has been steeped the longest and is transferred continuously in the direction of the corn most recently introduced. In this manner, the steep water having the highest concentration of

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solutes is used on corn just entering the system after which procedure the water is withdrawn and concentrated to a solid content of approximately 50%. This concentrate, crude corn steep liquor, may then either be combined with gluten and fibrous materials and sold as animal feed, or used for microbiological purposes, with or without further processing.

From the steeps the soft corn is transferred to the mill house where the multi-stage milling and the separation of the components of corn take place. After a coarse grind in an attrition mill, which consists of two large metal discs with metal teeth, the slurry is suspended in water and slowly passed through the germ separators. In these large troughs the germs, rich in oil, float to the top and are thus separated from the heavier materials which settle to the bottom. This residue, which consists of starch, gluten, and hull, is transferred to the reels, rotating coarse sieves of stainless steel, where the hulls are retained. To insure complete recovery of the valuable germs, the hulls are reground and passed again through the separators. The germs are moved over a battery of reels, washed free of adhering starch, and dehydrated, and the corn oil is extracted. The degerminated corn is partially freed of water in separator reels, and transferred to the Buhr mill which consists of two large granite stones, one on top of the other. The water removed at the separator reels contains considerable quantities of starch and gluten, which after being rid of coarse particles pass on directly to the table house. The degerminated corn is then reground in the Buhr mill, to a fineness sufficient to allow the separation of starch and gluten without at the same time also grinding the hulls and thus causing contamination of the starch with fibrous materials. Rotating reels are used to sieve out the coarse fiber, and rapidly moving silk shakers to remove the last pieces of fiber.

The mixture of starch and gluten is transferred onto the starch tables, which are flat-bottomed troughs, approximately 120 feet long, and slightly tilted so that the suspension will flow slowly towards the far end of the trough. The starch particles, being heavier than gluten, are deposited on the tables while the gluten flows off at the end of the table. The starch is washed off the tables, dewatered again by filtering on rotary filters, and finally dried. Washing and filtering may be repeated several times to remove all soluble products from the starch. Centrifuges are coming into vogue now and may eventually replace "tabling" as a means of separating the starch from the gluten. The gluten is recovered by allowing the liquor to stand in large tanks, the so-called gluten settlers, until it settles, and by filtering and drying the settled material. Figure 1 diagrams the various steps of the process. It is important to remember that large volumes of water are used, approximately 30 to 50 gallons per bushel of corn, and that much of this water is recovered from the filters and settling tanks and recycled in the process. Ideally, fresh water is introduced only for the final washing of the separated starch, and withdrawn only to the steeps, but such a multistage process is not easily kept in complete water balance (Cf. 20). Irregularities in procedure occur often, changing the character of the corn steep liquor.

During the steeping period and during the other phases of the process, there occurs an active natural fermentation, essentially lactic in nature. In spite of

the low pH, 3.8 to 4.5, the total viable count of organisms frequently runs into billions per ml. Variations of the order given in table 1 have been noted. In

## GENERAL FLOWSHEET

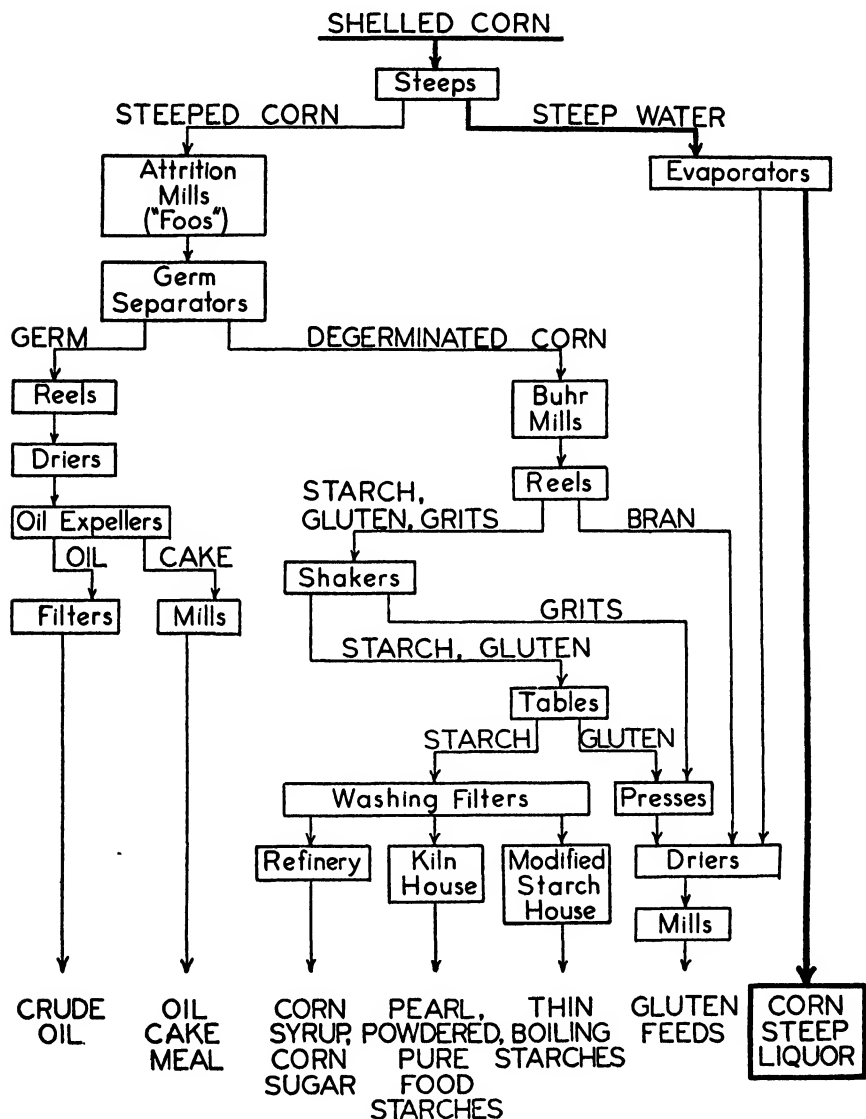


FIG. 1. GENERAL FLOWSHEET OF THE CORN WET-MILLING PROCESS

The flow of water is counter-current to the flow of materials as shown in the diagram. To maintain the water balance in the plant, water may be in process and storage for as long as two weeks. The water in the evaporator may come from any stage of the process.

general, the lowest microbial counts are found in process water containing the highest amount of  $\text{SO}_2$ , while the highest counts are found in the oldest process

water which is used on the most recent corn. In addition to the thermophilic bacteria, which are nearly all rods, other types have also been isolated by incubation at lower temperatures, including a variety of cocci. The predominant organisms, however, are thermophilic lactics, including both spore-formers allied to the "flat-sours", and representatives of the genus *Lactobacillus*. It should be pointed out that the lactic acid fermentation is not only important in the manufacture of starch but also in that of corn steep liquor of a quality desirable for microbiological purposes. The corn proteins are relatively water-insoluble, but they do swell and dissolve to some extent in acidified water. A certain amount of acidity is therefore essential for the efficient separation of starch from gluten, and the lactic acid complements the action of  $\text{SO}_2$ . The solution of the corn proteins, however is not entirely chemical, but also involves bacterial enzymes,

TABLE 1  
*Microorganisms in corn steep water*

TYPE	TOTAL COUNT	
	numbers/ml	
Aerobic bacteria <sup>a</sup> .....	30,000–	1,000,000
Anaerobic bacteria <sup>b</sup> .....	5,000–	20,000
Microaerophilic bacteria <sup>c</sup> .....	10,000,000–	10,000,000,000
Yeasts <sup>d</sup> .....	10–	1,000,000

<sup>a</sup> Surface colonies on brom cresol purple-dextrose agar plates, at 45 C. Organisms of *Bacillus subtilis* and *Proteus vulgaris* type; flat-sours not counted.

<sup>b</sup> Colonies in Brewer's thioglycollate agar tubes, at 45 C.

<sup>c</sup> Colonies in tryptone-yeast extract-dextrose agar tubes, at 45 C; primarily lactics. A count as high as ten billions occurs only in steeping systems that are out of balance; a count of one billion is more common as the upper limit.

<sup>d</sup> Colonies on wort agar plates, at 30 C.

which catalyze the breakdown of the native proteins to smaller units. Owing to this bacterial activity not only can starch be more easily separated from gluten, but the resulting corn steep liquor is high in amino acids and polypeptides, which are excellent sources of nitrogen for almost all microorganisms. If the bacterial fermentation is to accomplish such purposes it must be regulated, at least to a certain extent, by the control of certain environmental factors. For example, the relatively high steeping temperature, in the presence of readily available carbohydrates, favors the lactic acid fermentation and thus keeps putrefaction and alcohol formation to a minimum.

Essentially then, corn steep liquor consists of concentrated corn solubles, which have been extracted during the steeping process at approximately pH 4 and at a temperature of 45 to 52 C in the presence of  $\text{SO}_2$  and an active lactic acid fermentation. In addition, it includes soluble materials collected at other stages of the milling or separation processes; since process water may be retained in the plant for approximately two weeks it is not surprising that additional fermentations occur at many points. The yeasts isolated from the steep water do not

have the morphology and cultural characteristics typical of organisms cultured for several transfers on wort agar media, and apparently do not multiply actively.<sup>2</sup> However, at other stages of the process, which are conducted at lower temperatures, normal yeast development is thought to occur. For example, in the gluten settling tank and thickeners, where temperatures may go as low as 30 C, a scum of film yeasts occurs. Killinger (28) has emphasized the prevalence of yeasts and yeast-like organisms in starch process water.

The degree of fermentation that the solids of corn steep liquor have undergone varies greatly from plant to plant, and considerably even in one plant from time to time. To a certain extent, this variation can be controlled,<sup>3</sup> but there is also an apparent seasonal variation in the microbial flora, which so far has resisted control.

### *Chemical composition*

The main disadvantage of corn steep liquor in microbiology is its variable composition. This variability may depend somewhat upon the type and condition of the corn but even more upon a multitude of variables involved in the processing of starch. On the other hand, corn steep liquor is an inexpensive alternative (4 to 6 cents per pound of liquor) to much more expensive materials, such as yeast extract and peptone.

Corn steep liquor has a pH of 3.7 to 4.1, a specific gravity of 1.25, and on proximate analysis a general composition as indicated in table 2. On the average 6.9% of the corn solids and 30% of the corn nitrogen are found in the steep liquor. Ninety-five per cent of all samples have a nitrogen content of 3.85 to 4.1% while only 5% contain nitrogen below or above these limits. Similarly the majority of samples contain 1.45 to 1.65% amino nitrogen and 0.15 to 0.30% volatile nitrogen, largely ammonia. The amino N/total Kjeldahl N ratio for 95% of all samples is from 0.38 to 0.40%, indicating that the nitrogenous compounds present are, to a large extent, amino acids and polypeptides. Low amino N/total Kjeldahl N ratios are encountered in samples that have not undergone a vigorous lactic acid fermentation. To illustrate: the lot giving the lowest ratio ever encountered in these laboratories, also showed 11% of reducing sugar (determined as glucose), 5.1% of lactic acid, 0.15% of volatile nitrogen, and 0.1% of volatile acid (determined as acetic acid). This indicates a steeping system badly out of balance and one likely to be accompanied by the formation of scaly deposits in the evaporators. This scale or precipitate, spoken of as

<sup>2</sup> On first isolation on wort agar the yeasts from the steeps often give pin-point colonies consisting of very small, coccus-like cells. On the second or repeated transfers the organisms attain normal size and shape. *Torulopsis* shows normal morphology and colonial development even on first isolation. Among the yeast genera present, as kindly identified by Dr. L. J. Wickerham of the Northern Regional Research Laboratories, were *Trichosporon*, *Torulopsis*, and *Mycoderma*.

<sup>3</sup> An interesting example of this is a patent by Kerr and Berlin (27) which claims a reduction of scaling in evaporators if a secondary fermentation is encouraged by addition of carbohydrates and aeration.

"liver", consists almost entirely of coagulated proteins and insoluble salts of calcium and magnesium. Under normal steeping practices the coagulable proteins are degraded to smaller fragments, the greater number of which are no longer heat coagulable, and the insoluble salts are kept in solution by the lactic acid. Highly fermented steep liquors, on the other hand, show not only a high amino N/total Kjeldahl N ratio (0.50) but also a low sugar content (less than 1 to 2%), a high concentration of lactic acid (13 to 15%), and higher concentrations of volatile nitrogen compounds (0.40%) and volatile acids (0.3%) than poorly fermented steep liquors.

According to Cardinal and Hedrick (5), over 95 per cent of the total nitrogen in steep liquor is accounted for, after hydrolysis, by ammonia and the following amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, iso-

TABLE 2  
*General analysis of corn steep liquor*

DETERMINATION	PER CENT	DETERMINATION	PER CENT
Water <sup>a</sup> .....	45 -55	Lactic acid <sup>a</sup> .....	5 -15
Total Kjeldahl-N <sup>a</sup> .....	2.7 - 4.5	Ash <sup>a</sup> .....	9 -10
Van Slyke Amino-N <sup>b</sup> .....	1.0 - 1.8	Volatile acids <sup>d</sup> (as acetic acid).....	0.1 - 0.3
Volatile-N <sup>c</sup> .....	0.15- 0.40	SO <sub>2</sub> <sup>d</sup> .....	0.009- 0.015
Free reducing sugar (as glucose) <sup>a</sup> .....	0.1 -11.0		

<sup>a</sup> Based on determinations made on 1000 different lots.

<sup>b</sup> " " " " " 50 " "

<sup>c</sup> " " " " " 15 " "

<sup>d</sup> " " " " " 10 " "

\* The Amino-N/Total Kjeldahl-N ratio ranges from 0.30 to 0.50.

leucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, and valine. More than one-quarter of the nitrogen is present as alanine.

Corn steep liquor contains considerable amounts of the B-complex vitamins, with the exception of thiamine, which is usually low or absent, probably because it is destroyed by the SO<sub>2</sub> treatment during steeping. Tanner, Pfeiffer, and Van Lanen (59) analyzed a corn steep liquor medium used in the penicillin industry and found the following vitamin content, recalculated on the basis of micrograms of vitamin/gm of wet corn steep liquor: riboflavin, 5; niacin, 819; pantothenic acid, 23.8; pyridoxine, 19.1; and biotin, 0.125. Their data agree closely with our values: vitamins A, D, E, K, C and thiamine, usually 0; riboflavin, 5 to 10; pyridoxine, 25 to 400. The inositol content is uniformly constant and at least 1 mg/gm of steep liquor. Approximately half of the inositol occurs as phytin.

Table 3 presents information on the composition of corn steep liquor ash. Some of the discrepancies between the values listed may be due to the use of different analytical procedures rather than to actual differences in composition. Obviously the ash of corn steep liquor contains a wealth of mineral nutrients;

TABLE 3

*Composition of the ash of corn steep liquor*

ELEMENT	I % OF DRY MATTER	II % OF DRY MATTER
Al		0.032
Ca	0.5 -1.5	
Cd		0.0029
Cu	0 -0.001	0.0033
Fe	0.01 -0.05	0.052
Pb		0.055
Mg	0.5 -1.0	1.05
Mn	0.004	0.012
Mo		0.0006
P	2.0-3.0	2.75
K	1.0-2.0	
S	0.34	
Zn	0.005	0.0005

I = range of quantitative determinations made on 10 different lots.

II = data on a single lot; from Perlman (48).

A semi-quantitative spectroscopic analysis on a single lot, given by Koffler, Knight, and Frazier (33) indicates the presence of the following elements: Al, As, B, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Ni, P, K, Si, Ag, Sn, W, Zn. The following metals were not detected: Sb, Be, Bi, Cd, Cb, Ce, Au, La, Hg, Pt, Sr, Ta, Ti, V, and Zr. Another spectroscopic analysis, also on a single lot, is presented by Cook, Tulloch, Brown, and Brodie (16). These workers found the presence of the following elements: Ba, Pb, Mo, Ni, Rb, Sn, V, and Zn; the following were absent: Cr, Co, Li, and Ag.

TABLE 4

*Some uses of corn steep liquor in microbiology*

USED IN THE PRODUCTION OF
Yeast food (1)
Yeast (62)
Leavened dough-products (64)
Yeast stimulant (63)
Organic acids from cellulosic material (35)
Beer (18)
Bread dough (49)
Brewing adjunct (65)
Sorbose by <i>Acetobacter suboxydans</i> (66)
Ketogluconic acids by <i>Acetobacter suboxydans</i> and an unnamed organism (58)
Gluconic acid by <i>Aspergillus niger</i> (47)
Itaconic acid by <i>Aspergillus terreus</i> (40, 41, 42, 43)
Penicillin by <i>Penicillium notatum-chrysogenum</i> (44, 45)
Pentonic acids by <i>Pseudomonas</i> (39)
Riboflavin by <i>Ashbya gossypii</i> (60)
Subtilin by <i>Bacillus subtilis</i> (37)
Amylase by <i>Aspergillus niger</i> (36)

especially calcium, iron, magnesium, phosphorus, and potassium occur in high concentrations.

### *Use in Nutrient Media*

Although Behr (1), who is credited with the invention of much of the modern wet milling process, suggested corn steep liquor as a nutrient for microorganisms as early as 1909, its use in microbiology until recently was limited to yeast fermentations. However, workers at the Fermentation Laboratories of the United States Department of Agriculture and others have advocated the use of corn steep liquor in other fermentations so enthusiastically that its usefulness as a general substrate for microorganisms is being recognized. It hardly needs to be mentioned that corn steep liquor has contributed, in a large measure, to the rapid development of the penicillin industry. Table 4 which gives the highlights in the uses of corn steep as a component of nutrient media should be indicative that the potentialities of this material are beginning to be realized.

In the laboratory, corn steep liquor may serve either as a supplement to replace extracts, or as the main source of nitrogen and carbon for all microorganisms except fastidious pathogens. In general, any organism capable of growing well on simple media containing beef extract and peptone will grow on media containing only corn steep liquor. We have successfully grown the following organisms on one or several of the media mentioned below:

Bacteria	Yeasts and Yeast-like Fungi	Molds
<i>Acetobacter suboxydans</i>	<i>Candida Guilliermondi</i>	<i>Aspergillus flavus</i>
<i>Bacillus macerans</i>	<i>Candida lipolytica</i>	<i>Aspergillus niger</i>
<i>Bacillus subtilis</i>	<i>Endomycopsis fibuliger</i>	<i>Aspergillus oryzae</i>
<i>Lactobacillus arabinosus</i>	<i>Saccharomyces cerevisiae</i>	<i>Eremothecium ashbyii</i>
<i>Lactobacillus brevis</i>	<i>Schizosaccharomyces octo-</i>	<i>Mucor boudard</i>
<i>Lactobacillus casei</i>	<i>sporus</i>	<i>Mucor mucedo</i>
<i>Lactobacillus delbrueckii</i>	<i>Torulopsis utilis</i>	<i>Neurospora crassa</i>
<i>Lactobacillus fermenti</i>	<i>Zygosaccharomyces japo-</i>	<i>Oospora lactis</i>
<i>Leuconostoc mesenteroides</i>	<i>nicus</i>	<i>Penicillium notatum</i>
<i>Micrococcus pyogenes</i> var.	<i>Zygosaccharomyces mellis</i>	<i>Penicillium chrysogenum</i>
<i>aureus</i>	<i>Zygosaccharomyces Nuss-</i>	<i>Phycomyces</i>
<i>Pseudomonas aeruginosa</i>	<i>baumeri</i>	<i>Rhizopus japonicus</i>
<i>Streptococcus faecalis</i>	<i>Zygosaccharomyces Richeri</i>	
<i>Streptococcus lactis</i>		
<i>Streptomyces cellulosa</i>		
<i>Streptomyces coelicolor</i>		
<i>Streptomyces diastaticus</i>		
<i>Streptomyces flavovirens</i>		
<i>Streptomyces griseus</i>		
<i>Streptomyces microflavus</i>		
<i>Streptomyces olivaceus</i>		

If clear media are desired, preliminary treatment of the crude liquor becomes necessary. A good practice is to adjust the liquor with water until it contains from 15 to 20% solids, raise the pH to 8 with concentrated KOH, autoclave for an hour, and cool and filter. The filtrate then may be reconcentrated or re-

frigerated. For certain purposes, such as penicillin production, the quality of the steep liquor is impaired by this treatment. Refrigeration of corn steep liquor is advisable to prevent spoilage by yeasts.

The following are some representative media. For yeasts, 0.5% corn steep liquor solids, with sugar as desired. For bacteria, 1.0% corn steep liquor solids, 0.5% glucose, adjusted to pH 7.4, as a basal medium. This can be modified in various ways, e.g., for the lactics, 1.0% corn steep liquor solids, 1.5% glucose, in 1.0% phosphate buffer at pH 7.0. Also the following combinations have proved valuable after adjustment to the desired pH: *a*, 0.5% corn steep liquor solids and 0.5% tryptone for bacteria; and *b*, 1.0% corn steep liquor solids and 0.3% yeast extract, for bacteria and yeasts. For media used in industrial processes, the references cited in table 4 should be consulted.

#### *Use in the Production of Penicillin*

By far the most important application of corn steep liquor in microbiology was discovered by Moyer and Coghill (44) who noticed that the addition of corn steep liquor to a modified Czapek-Dox solution of mineral salts greatly increased penicillin yields. This discovery encouraged many efforts to isolate the active component which endows corn steep liquor with the remarkable ability to stimulate the biosynthesis of penicillin by organisms of the *Penicillium notatum-chrysogenum* group. Since, according to studies on the metabolism of these organisms, the biological function of steep liquor cannot be ascribed to a single factor but to a group of factors (31, 48, 19, 25, 34, 45, 54, 55, 30), such attempts were unsuccessful.

Penicillin appears to be synthesized by the live, active mold and in largest quantities when the pH is between 7.0 and 8.0<sup>4</sup>; therefore, conditions which tend to maintain the mycelium metabolically active and the pH of the medium within the optimum range usually favor maximum penicillin yields. Assuming that certain environmental factors, such as temperature and aeration are kept optimum, these conditions can be provided by a balanced mixture of a variety of nutrients, which includes a readily available source of carbon and nitrogen, a slowly fermentable carbohydrate, and an effective buffer system. The first allows rapid initial formation of the mycelium, the second serves as a slowly available store of energy, which can be tapped throughout the entire period of growth, and the third obviously aids in the maintenance of the pH values within a desired range. Other nutritional factors, such as mineral elements are of course also essential, and will be mentioned later.

Studies of the metabolic changes that occur during the growth of penicillin-producing molds reveal a pattern which is characteristic for good penicillin yields; conditions unfavorable for optimum penicillin production ordinarily find their expression in deviation from the typical metabolic pattern. In this manner it can be demonstrated that corn steep liquor, fortunately enough, is one of the few inexpensive and readily available materials which include a desirable balance

<sup>4</sup> For certain synthetic media the pH best for penicillin production has been found to be 7.3 as compared to 6.8 which seemed optimum for the growth phase of the mold (24).



of as many of the essential factors as possible. Lactic acid is a readily available source of carbon for the penicillin-producing molds; amino acids and polypeptides serve as readily available sources of both carbon and nitrogen and also as buffer systems; the ash of corn steep liquor readily supplies the need of the organism for mineral elements. The only important constituent lacking is a slowly available source of energy, which in the media commonly used in the industry, is furnished as lactose. To obtain maximum penicillin yields the exact composition of corn steep liquor-lactose media has to be varied with the degree of aeration. This depends on whether surface cultures, shake flask fermentations, or large volume tank fermentations are employed (4). The media for large-scale production usually also contain 1.0%  $\text{CaCO}_3$  to make the reaction of the medium less acid.

The fact that synthetic media can be employed in the production of penicillin supports the view that a combination of factors rather than a single factor accounts for the excellence of steep liquor. For example, glucose and acetic acid may be substituted for the readily available carbon compounds of corn steep liquor, and ammonium salts for the readily available nitrogen compounds. Phosphates serve as buffers; metabolism of acetate controls the pH of certain synthetic media during the primary growth phase, while the pH during the subsequent penicillin-forming phase is greatly influenced by the rate of lactate utilization. Salt solutions containing S, Fe, K, Mg, Zn, Cu, Mn, and Ca in addition to P, may replace the ash fraction of corn steep liquor. Lactose is added as the slowly fermentable carbohydrate. The work on synthetic media done at the Pennsylvania State College (56) and the University of Wisconsin (24) should be consulted for further details.

In addition to the non-specific factors already mentioned, specific precursors of penicillin may occur in steep liquor. Inspection of the structural formulae of the various penicillins (6) suggests a considerable number of compounds which potentially may serve as precursors. However, only a few, such as phenylethylamine and tyramine, which are decarboxylation products of phenylalanine and tyrosine, respectively, have been demonstrated to occur in corn steep liquor (2, 3, 17). Of course, it is now an established industrial practice to stimulate the biosynthesis of penicillin by furnishing precursors of various chemical types to the mold (22, 57, 24, 46, 3).

The early efforts to elucidate the role of corn steep liquor usually were frustrated because they involved adding fractions of steep liquor, or known compounds to basal media from which corn steep liquor had been omitted. Since these basal media ordinarily consisted only of a modified Czapek-Dox solution and lactose, they contained in the absence of corn steep liquor neither enough nitrogen nor phosphorus to permit adequate penicillin production. For example Moyer and Coghill (44) found that neither trace elements nor the redissolved ash of corn steep liquor, nor growth factors, nor amino acids when added to such a basal medium could replace corn steep liquor in its ability to stimulate penicillin production. On the other hand Knight and Frazier (29) showed that the ash of corn steep liquor was stimulatory to penicillin production. These workers

used a basal medium which was adequate in all respects except, as shown later by Koffler, Knight and Frazier (33), in its content of iron and phosphorus. Under these conditions the ash appeared to be the important constituent of corn steep liquor because it supplied the needed iron and phosphorus. Similarly, White, Krampitz, and Werkman (67) claimed that a mixture of histidine, arginine, glutamic acid and succinic acid was responsible, at least in part, for the stimulatory activity of corn steep liquor. It would seem now that these acids primarily functioned as readily available sources of carbon and nitrogen in a medium deficient in these factors. All these reports support the thesis that in order to test the essentiality to penicillin production of one nutritional or other factor, all other conditions should be optimal.

The work of Cook and his associates (8, 9, 10, 11, 12, 13, 14, 15, 16) is of interest in this connection. These workers claimed that aqueous extracts of peas, when added to a basal medium, stimulated penicillin yields. After considerable chemical work, which included careful fractionation of the stimulatory extract, they decided that diverse nitrogen and carbon compounds had the same stimulatory effect. Apparently their basal medium was deficient in readily available sources of carbon and nitrogen, and pea extracts influenced the well-being of the molds generally, rather than penicillin synthesis specifically.

The mineral nutrition of the mold also seems to be of great importance to the success of the penicillin fermentation. It is indeed fortunate that corn steep liquor contains so many mineral elements, and therefore adequately supplies the mold with the mineral elements necessary to penicillin formation. Theoretically, mineral elements could serve several functions in penicillin production. Some elements may function *directly* as constituents of enzymes that are essential to mold metabolism in general, or to penicillin synthesis in particular. Or, some mineral elements may act *indirectly* because they protect either the mold from the harmful effects of toxic elements or penicillin from the destruction catalyzed by other elements. Hutner (23) holds the general view that many elements are considered essential nutrients because they form precipitates in dilute media and thereby remove toxic elements by precipitation or adsorption. Of interest are the recent papers by Pulvertaft and Yudkin (53) and Pratt (50, 51, 52) who claim that phosphates specifically stabilize penicillin by effects other than those which they exert as buffers. Pratt suggests phosphorylation of the penicillin molecule. It is also possible that harmful elements are removed as insoluble phosphates. In certain synthetic media, the level of phosphates required to give optimum growth of the mold is lower than the amount to give optimum penicillin yields. This may be explained possibly by the protective effect that phosphates exert on the penicillin. Since at any one time penicillin "yields" are influenced by synthesis and destruction of penicillin (processes that are thought to occur to varying degrees simultaneously), the high content of phosphates in steep liquor may be of additional advantage. Perhaps the increase in penicillin yields, observed in certain cases, when supplements of boric acid or citric acid were added to corn steep liquor-lactose media (32, 44) might be explained similarly. Thomas (61) and Hahn (21) recently have demon-

strated that penicillin is considerably more stable in citrate buffers than in saline or phosphate solutions. It is conceivable that boric acid and citric acid appeared to stimulate penicillin yields because they combine with ions which otherwise would be harmful to the production of penicillin either by poisoning the mold or by catalyzing the destruction of penicillin.

Interestingly enough, better penicillin yields can be obtained from submerged cultures when  $\text{CaCO}_3$  is used to neutralize the otherwise acid corn steep liquor medium rather than KOH (45) or NaOH (19). Could these higher yields be ascribed to the fact that  $\text{CaCO}_3$  also adsorbed deleterious materials in addition to raising the pH? In spite of any toxic components, which steep liquor may contain, there is little doubt that it has become a valuable source of nutrients for microorganisms in industry and the laboratory.

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